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METABOLISM OF CARBAMATE INSECTICIDES

KENTUCKY UNIVERSITY

PREPARED FOR
ENVIRONMENTAL PROTECTION AGENCY

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The metabolic fate of aldicarb, carbaryl, and carbofuran was investigated in a variety of biological systems. In addition, the effects of other insecticides and certain monoamine oxidase inhibitors on carbaryl metabolism in rats was studied. The fate of 3-hydroxy carbofuran, its glucoside and glucuronide, and naphthyl glucoside in rats was determined. Using 1-naphthol as a model compound, in vitro methods were developed to study mechanisms of glycosylation in insects and mammals. The glucosides of 4- and 5-hydroxy carbaryl were prepared chemically and their acute toxicity to mice compared to the aglycones. Results of these studies showed that carbamate insecticides are metabolized initially by hydrolytic- and oxidative-type reactions and the resulting products are then almost totally conjugated. These conjugated products constitute the majority of the terminal residues of carbamates in both animals and plants.

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METABOLISM OF CARBAMATE INSECTICIDES

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ABSTRACT

The metabolism of selected carbamate insecticides and certain of their key metabolites are reported. Aldicarb was studied in lactating cows, laying hens, and in boll weevils and houseflies. Carbaryl was investigated in lactating cows and in soil. Carbofuran was studied in houseflies.

Effects of exposure to mixtures of pesticides on the metabolism of carbaryl were determined. The fate of the carbaryl was compared to that in animals exposed to carbaryl alone. Similar studies were conducted where monoamine oxidase inhibitors were given to the rats. Aldicarb, an oxime carbamate, was evaluated for its effect on the toxicity of methyl parathion to rats.

Radioactive 3-hydroxycarbofuran was biosynthesized and its metabolism investigated in rats and bean plants. Similar studies were performed with its glucoside and glucuronide and with 1-naphthol and its glucoside conjugate.

Mechanisms of glycosylation were studied in a variety of animal species. Successful chemical syntheses of the glucosides of 1-naphthol, 4-hydroxycarbaryl and 5-hydroxycarbaryl were accomplished. Acute toxicity of the carbamate derivatives to rats was determined.

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CONTENTS

Page

List of Tables vi

Acknowledgments x

Sections

I Conclusions 1

II Recommendations 2

III Experimental 3

- Introduction 3
- Metabolism 3
  - Aldicarb in Lactating Cows 4
  - Aldicarb in Laying Hens 31
  - Aldicarb in Boll Weevils and Houseflies 51
  - Carbaryl in Lactating Cows 56
  - Carbaryl in Soils 83
  - Carbofuran in Houseflies 113
- Fate of Carbamate Metabolites 120
  - 1-Naphthyl Glucoside in Rats 120
  - 3-Hydroxy Carbofuran and its Glycosides in Rats 130
- Interactions 139
  - Effect of Aldicarb on Methyl Parathion Toxicity 139
  - Modification of Carbaryl Metabolism with MAOI's 144
  - Influence of Insecticides on Carbaryl Metabolism 156
- Conjugation 174
  - Mechanism in Rats 174
  - Mechanism in Insects 189
  - Factors Influencing Conjugation 199
  - Synthesis of Glycosides 214

IV References 226

V Publications 234

VI Summary 236

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TABLES

No. Page

1. Aldicarb and its metabolites 24
2. Aldicarb rates and feed consumption 25
3. Elimination of radioactivity by cows fed alidcarb-^{14}C 26
4. Aldicarb-^{14}C equivalents in milk 27
5. Aldicarb-^{14}C equivalents in urine 28
6. Separation of water-soluble aldicarb-^{14}C metabolites 29
7. ^{14}C-Residues in tissues of cows fed aldicarb-^{14}C 30
8. Treatment rates of aldicarb-^{14}C to laying hens 42
9. Nature of aldicarb-^{14}C equivalents in feces of hens 43
10. Elimination of aldicarb-^{14}C residues in the feces of hens 44
11. Aldicarb-^{14}C residues in feces of hens 45
12. Ppm aldicarb equivalents in tissues and eggs of hens 46
13. Residues in tissues and eggs of hens treated with radioactive aldicarb 47
14. Residues in eggs of hens aldicarb in the diet 48
15. Ppm aldicarb-^{14}C equivalents in tissues of hens fed aldicarb in the diet 49
16. Residues in tissues of hens fed aldicarb in the diet 50
17. Distribution and excretion of aldicarb in boll weevils and houseflies 55
18. Study plan for carbaryl-naphthyl-^{14}C cow feeding experiment 74
19. Chemical nature of carbaryl metabolites in cow's milk 75
20. Total carbaryl-^{14}C equivalents in cow tissues 76
21. ^{14}C-Residues in cow tissues after feeding with carbaryl-^{14}C 77
22. Centrifugal fractionation of ^{14}C-residues in the liver and kidney of a cow fed carbaryl 78
23. Extraction characteristics of ^{14}C-residues in acid-treated supernatant of liver and kidney 79
24. Extraction characteristics of ^{14}C-residues in acid-treated pellet of liver and kidney 80

TABLES - cont.

No.		Page
25.	^{14}C-Residues in the precipitate of a 15,000g supernatant of liver	81
26.	Precipitation, and dialysis of a 15,000g supernatant of kidney	82
27.	Radioactivity of soils fortified with carbaryl-1-^{14}C and incubated at 27°C, 85% R.H.	102
28.	Radioactivity in soils fortified with carbaryl-1-^{14}C and incubated at 27°C, 85% R.H.	103
29.	Radioactivity of carbaryl-treated soils fortified with polar water-soluble metabolites of carbaryl-^{14}C	104
30.	Radioactivity of soils fortified with polar water-soluble metabolites of carbaryl-^{14}C incubated at 27°C, 85% R.H.	105
31.	Fate of carbaryl-naphthyl-1-^{14}C and 1-naphthol-^{14}C in carbaryl-treated soil	106
32.	Radioactivity of acetone and barium hydroxide trap solutions	107
33.	$^{14}CO_2$ from carbaryl-^{14}C and 1-naphthol-^{14}C during incubation with soil	108
34.	Carbaryl-^{14}C inoculated with suspension of mixed pesticide-treated soil	109
35.	Carbaryl-1-^{14}C inoculated with carbaryl-treated soil suspensions or fungal isolates	110
36.	Carbaryl-^{14}C inoculated with bacterial isolates	111
37.	Nutrient broth with carbaryl-^{14}C inoculated with bacterial isolates	112
38.	Nature and magnitude of carbofuran in the body and excreta of houseflies	119
39.	Nature of residues in the 0-24 hour urine of rats treated orally with 1-naphthol and 1-naphthyl glucoside	129
40.	Influence of carbofuran on its metabolism by rat liver	135

TABLES - cont.

No. Page

41. Effect of substrate concentrations on glucuronidation 136
42. Effect of incubation periods on the glucuronide and glucoside conjugation 137
43. Partitioning characteristics of metabolites in the urine of rats treated orally with 3-OH-carbofuran or its glucuronide or glucoside derivatives 138
44. Effect of aldicarb, aldicarb oxime, 2-PAM and atropine on the acute toxicity of methyl parathion to mice 143
45. Effect of monoamine oxidase inhibitors on the excretion of carbaryl-^{14}C by rats 152
46. Fate of carbaryl-^{14}C in rats which received phenelzine in the drinking water 153
47. Nature of carbaryl-^{14}C metabolites in urine of normal and phenelzine-treated rats 154
48. Metabolism and excretion of naphthol-^{14}C when administered with phenelzine 155
49. Effects of carbofuran and Ruelene on the excretion of carbaryl-^{14}C by rats 166
50. Excretory pattern of carbaryl by rats fed a diet containing carbofuran, Ruelene or coumaphos 167
51. Nature of radioactivity in the urine of rats treated with carbaryl-^{14}C alone and with either carbofuran or Ruelene 168
52. Weight and microsomal protein content of livers from rats exposed to carbaryl, carbofuran and DDT 169
53. In vivo metabolic activity in the microsomal fractions of liver and kidney of rats fed carbaryl, and DDT for 40 days 170
54. TPNH oxidase activity in the microsomal fractions of livers from rats treated IP with carbaryl and DDT daily for up to 5 days 171

TABLES - cont.

No. Page

55. The effect of pre-exposure to DDT on the nature and magnitude of carbaryl-^{14}C metabolites in the urine of rats 172
56. Excretion and metabolism of naphthol-1-^{14}C by rats given daily injections of DDT for 5 days 173
57. Importance of conjugation in the metabolism of carbamate insecticides in rats 185
58. Effect of insecticide synergists on conjugation of 1-naphthol by rat liver microsomes and intestine enzymes 186
59. Inhibition of rat liver microsomal epoxidation of aldrin by certain insecticide synergists 187
60. Effect of reduced glucuronidation on the total metabolism of carbaryl-^{14}C 188
61. Reaction mixture used for the in vitro conjugation of 1-naphthol by tobacco hornworm midgut enzyme 196
62. 1-Naphthol glucosylation of whole body extracts of adult houseflies and tobacco hornworm larvae 197
63. Sensitivity of glucosylation and epoxidation enzymes to insecticide synergists 198
64. Glucosylation by the 5,000g supernatant of different insects and insect tissues 208
65. Glucosylation by subcellular fractions of a housefly homogenate 209
66. The effect of age and sex on conjugation by susceptible and resistant houseflies 210
67. Fate of topically applied 1-naphthol-1-^{14}C in susceptible and resistant houseflies 211
68. Conjugation by the microsomal fractions of livers from different animals 212
69. Glucuronyltransferase activity in various rat tissues 213
70. The toxicity of 4- and 5-hydroxycarbaryl and their respective glucosides to mice 225

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I CONCLUSIONS

In animals, the carbamate insecticides, aldicarb, carbaryl, and carbofuran are rapidly metabolized and excreted from the body. Continuous exposure of animals to the pesticides added to the diet do not result in the accumulation of high levels of residues. However, metabolites containing the carbamate moiety, and therefore considered as potentially toxic, do exist in the meat, milk and eggs of treated animals.

Glucoside derivatives of carbamate insecticide metabolites formed in plants do not behave in the animal system in the same manner as the aglycone. Cleavage of the glucoside linkage yields the aglycone which is further metabolized by conjugation to form glucuronides, sulfates, etc. Little, if any, oxidation of the sugar moiety takes place to form the corresponding glucuronide. Studies with naphthyl glucoside showed that the glucosidic bond was stable enough so that about 20% of an oral dose was eliminated, intact, in the urine.

Mechanisms of glycosylation of carbamates and other chemicals can be effectively investigated using in vitro techniques developed in this program. Also, it is now possible to critically evaluate the chemical and toxicological properties of glycosides of certain carbamate metabolites. The glucosides of 4- and 5-hydroxycarbaryl were chemically synthesized in quantities suitable for such studies.

II RECOMMENDATIONS

Emphasis should be placed on developing practical analytical methods for monitoring residues of toxic carbamate metabolites. Metabolites containing the carbamic acid ester exist both in the free and conjugated forms. However, these metabolites often go undetected because of improper extraction techniques and/or of inadequate methods of detection.

Because the majority of the terminal residues of carbamate insecticides in animals and plants are water-soluble and/or unextracted from the substrate, the significance of these materials in animals must be determined. Attempts should be made to study the toxicology of each product individually. However, it will be years before this is technically feasible. Therefore, the unknown residues should be generally characterized: organo-soluble, water-soluble, unextracted, carbamate vs. hydrolysis products, etc. Then, the compounds should be separated accordingly and their fate in animals evaluated. Metabolites stored or accumulated in the body should be generated in sufficient quantities to allow 90-day feeding studies to be performed. If the "no-effect" level is too low, the continued use of the parent insecticide must be reconsidered.

The development of the carbamate insecticides as commercial pest control agents should be encouraged. As a group, the compounds have exhibited (1) effective insect control, (2) relatively short residual life in the environment, (3) rapid and almost quantitative excretion from the animal body, and (4) terminal residues that are polar in nature and are formed by chemical processes normally considered as metabolic detoxication. Unless new and better insecticides are made available within the next 3 to 5 years, it is likely that this country will suffer insect-related catastrophes of greater consequent than ever experienced by mankind. A well-devised plan to facilitate the development of the carbamate insecticides could prevent such an occurrence.

III EXPERIMENTAL

INTRODUCTION

The principal investigator began work on this project in 1965. At that time, the research was funded by the National Institute of Health and was supported initially for a 3-year period. The first progress report was submitted April 3, 1967 covering the period from January 1, 1965 through December 31, 1967. The report, 266 pages, was identified as NIH Research Project ES-00085. Continued funding was requested and approved, and the metabolism of carbamate insecticides was further investigated. The next progress report, 246 pages, was submitted December 10, 1969 covering the period of January 1, 1968 through December 31, 1969. Because of changes within NIH, the project was identified as Food and Drug Administration Grant Number FD-00273. A renewal of the grant was requested and approved for the period of September 1, 1970 through August 31, 1973. On May 21, 1971, the grant number was changed to EP00828.

Basically, the main objective of the research proposed in 1964 has not changed. This objective, namely to determine the metabolic fate of carbamate insecticides in various biological organisms, was believed essential if the carbamates were to become major agents of pest control in this country. It is now well established that commercial development of the carbamates did continue and are vital to agricultural and health programs throughout the world. Research funded by the current project has been instrumental in the development of the carbamates. Metabolic pathways have been established for many of these insecticides in a variety of animal and plant species. These data have been relied upon heavily by regulatory officials who must determine if the compounds can be safely used.

Initially, most of the research into the metabolism of carbamate insecticides dealt with metabolites of a "free" nature. These were materials formed by oxidative and hydrolytic mechanisms and were generally characterized as "organo-soluble" metabolites. Subsequent work demonstrated that the free metabolites were often converted to other materials having vastly different extraction and partitioning characteristics. These metabolites are referred to as "water-solubles" and/or "bound" residues depending upon their behavior during analyses of the substrate. Little is known about the chemical and toxicological properties of the water-solubles and bound metabolites. Consequently, much of our efforts have been in this area during the past 2 years.

In this report, the research which has been conducted since September 1, 1970 is included in 4 different categories. First, studies designed to determine the metabolic fate of carbamate insecticides in a specified organism will be presented. Second, work conducted on the fate of selected carbamate metabolites will be considered. Third, results of studies to determine the interactions of pesticides and/or some selected drugs on carbamate metabolism will be discussed. Fourth, a report will be presented covering studies of the mechanisms of glycosylation of carbamate materials, and its significance in the over-all metabolism of these toxicants.

METABOLISM

Aldicarb in Lactating Cows

An earlier report showed that when Aldicarb [2-methyl-2-methylthio) propionaldehyde O-(methylcarbamoyl) oxime], was administered to a lactating cow as a single oral dose, approximately 85% of the dose was eliminated from the body within 24 hours (Dorough and Ivie, 1968). Aldicarb equivalents in the milk were low, maximum of 62 p.p.b., and no Aldicarb per se was detected. The nature of the metabolites was similar to that reported for other animals and for plants. The data indicated that the rapid excretion of Aldicarb, primarily in the urine, by dairy animals would prevent the accumulation of residues in milk and tissues to appreciable levels. However, this could not be stated with certainty because continuous feeding of Aldicarb could alter its metabolic fate, possibly resulting in higher residues and/or ones different chemically from those found after a single treatment.

The need for long-term, continuous feeding studies stems largely from the behavior of the chlorinated hydrocarbon pesticides. Certain of these toxicants appear in milk even when very low levels are in the cow's feed. Furthermore, several weeks, and often several months, of feeding are necessary before the concentration of residues in the milk reaches a plateau. For example, heptachlor epoxide residues appeared to be still increasing at the end of a 35-day feeding period (Williams et al., 1964). With dieldrin, endrin, lindane, and DDT, the concentration of each residue in milk had reached a maximum at various times during the feeding period. The nature of these chemicals continues to influence the investigational format of other groups of pesticides, such as the carbamates, despite the fact that these newer products are different chemically, biologically, and metabolically. Obviously, a format based on the chlorinated hydrocarbons would not necessarily be appropriate for all other insecticides. In addition to establishing the fate of Aldicarb in cows when administered over a long period of time and correlating dosage rates to residue levels in meat and milk, the present investigation should be useful in determining experimental parameters most desirable for future studies using similar compounds.

The concentration of Aldicarb and/or its metabolites in mature forage crops treated with Aldicarb has not been reported. However, sufficient work has been done to indicate the type of metabolites that must be considered. Aldicarb is readily oxidized in cotton to its sulfoxide which is then slowly metabolized to the sulfone (Coppedge et al., 1967; Metcalf et al., 1966). When applied to the soil, maximum uptake of

Aldicarb-S^{35} by cotton plants occurred within 2 weeks, with no indication of significant uptake thereafter. After 6 weeks, Aldicarb sulfoxide and Aldicarb sulfone were present in mature leaves in about equal quantities. No Aldicarb was detected. These data demonstrate that the residues likely to be present in feeds would be Aldicarb sulfoxide and Aldicarb sulfone and that these products should be considered when evaluating the fate of Aldicarb residues in dairy animals.

Because Aldicarb sulfoxide is unstable in the pure form, Aldicarb was used in the present study. Based on the rapidity with which Aldicarb was converted to its sulfoxide in rats (Andrawes et al., 1967), a fate study of Aldicarb would yield results comparable to those obtained should Aldicarb sulfoxide be the administered compound. The rapid conversion of Aldicarb to Aldicarb sulfoxide in a lactating cow also was noted (Dorough and Ivie, 1968). Even in the urine, which contained over 90% of the dose given, Aldicarb was not detected. However, Aldicarb sulfoxide accounted for over 50% of the total Aldicarb equivalents in urine collected only 3 hours after treatment. Thus, the 1 to 1 molar ratio of Aldicarb and Aldicarb sulfone administered to the cows in the current study was intended to represent, as closely as possible, actual residues that could be consumed by cows if fed crops treated with Aldicarb for insect control.

Radioactive Aldicarb was used in an attempt to obtain a complete picutre of its metabolic fate in dairy cows. Admittedly, total detectable residues, and consequently the number of unknown metabolites, are probably increased over those observed had more selective analytical methods been employed. However, with selective methods, the scope of any metabolic fate or residue study is limited to a predetermined set of conditions. There is little likelihood that products of an unpredictable nature would be detected. This could result in toxicologically significant metabolites being overlooked in the environment and/or in products destined for human consumption. Radiotracer techniques do not assure the identity of all residues but they do assure that the presence of most metabolites is recognized. This way, it is possible for them to be evaluated as to general chemical nature, concentration, and detectability. Depending upon these factors, judgment may be made as to the need for additional characterization.

Methods

Animals - Holstein cows purchased from a commercial dairy were used in this study. One animal was used in a pilot study to determine the effect of the high Aldicarb dose on the general health of the cow. The animal weighed 645 kg. and was in the latter stage of lactation. Three additional cows were used in the radioactive Aldicarb feeding study. Each had calved 6 to 8 weeks previously and was in peak milk production. The cow fed the low level of Aldicarb weighed 470 kg., that fed the medium level 500 kg., and the one fed the high level weighed 518 kg. at the beginning of the experiment. It was incidental that the weights of the cows increased with the dosage rates. In fact, the animals were placed in designated stalls prior to being weighed and by persons unfamiliar with the fate of the animals.

The animals were held in metabolism stalls and a 12-hour milking schedule was maintained. Milking was done by machine with separate apparatus used for each cow to minimize chances of cross contamination. The close proximity of the cows proved to be far superior to the single isolated animal situation used in earlier investigations. There was little sign of nervousness by the cows, and they responded to handling in a normal manner. More important, there was no decline in milk production owing to confinement as had often been the case with an isolated cow.

For the first 10 days of the study, during which the feces and urine were not collected, the cows were exercised daily. However, the cows were held in constant confinement during the next 14 days while being fed radioactive Aldicarb and while the urine and feces were collected separately.

The urine was collected by attaching a flexible vinyl hose, 1 inch in diameter, to the vulva of the cow. First, the hose was fixed to a triangular pad constructed of vinyl tape in such a manner that the opening of the hose could be positioned directly over the vulva. Oils on the skin surrounding the vulva were removed by washing with ether, and then a layer of contact cement was applied. Contact cement was applied to the triangular pad also. When the cement on both surfaces was dry, the pad was attached to the vulva. To prevent the hose from falling beneath the animal when she was in a prone position, it was suspended from the ceiling with rubber tubing so that it was held away from the animal and about 12 inches from the floor at the lowest point. Collecting the urine by this method created no apparent problem for the cows and proved sufficiently stable so that only one hose had to be repaired during the 14-day test period.

To take samples of blood at frequent intervals without exciting the cows, an intravenous catheter was inserted into the jugular vein of each animal before treatment began. The catheter was equipped with a receptacle for a standard syringe with which the blood was withdrawn. As there was no pain associated with sampling the blood using this technique, it was unnecessary to restrain the animals.

At the morning and evening milkings each cow was given 12 pounds of grain. Water and alfalfa hay were provided ab libitum. The amount of feed consumed by each animal was recorded daily.

Treatment and Sampling - Aldicarb-S-methyl-C^{14} and Aldicarb sulfone-S-methyl-C^{14}, both having a specific activity of 5 mCi. per mmole, were obtained from the Union Carbide Chemicals Co. as were nonradioactive forms of these chemicals and metabolite standards (Table 1). Equal molar quantities of Aldicarb and Aldicarb sulfone were dissolved in acetone and appropriate aliquots of the solution transferred to a gelatin capsule containing crushed grain. The capsules were administered to the cows with a balling gun twice daily at 12-hour intervals, one at the morning milking and one at the evening milking. For each treatment, feeding was begun and then, after several minutes, was interrupted briefly when the cow was given the capsule. The cows were milked immediately thereafter.

Dosage rates of Aldicarb were calculated on the basis of an anticipated total feed intake of 50 pounds per cow per day. For example, the cow receiving 1.2 p.p.m. Aldicarb equivalents in the diet was given 0.036 mmoles, 6.8 mg., of Aldicarb and 0.036 mmoles, 7.9 mg., of Aldicarb sulfone at each feeding.

The cow used in the pilot study was given nonradioactive Aldicarb and Aldicarb sulfone at the rate equivalent to 1.2 p.p.m. of Aldicarb in the diet for a total of 10 days. Animals in the dosage series were fed nonradioactive insecticide for 10 days and then the radioactive products for an additional 14 days. In all cases, treatment was continuous throughout the indicated time periods.

Blood was taken daily from each cow while on the Aldicarb treatment. Samples were withdrawn 6 hours after the morning milking and were immediately assayed for plasma and red blood cell (RBC) cholinesterase activity. Blood from animals fed radioactive Aldicarb was also radioassayed to determine the total Aldicarb equivalents present. Milk, urine, and feces were collected at 12-hour intervals, weighed, and then frozen until analyzed. Eighteen hours after the last radioactive Aldicarb treatment, the cows were slaughtered and tissue samples removed for analysis.

Assay Procedure - For all radioactive measurements reported herein, a Packard Tri-Carb Model 3365 instrument was used. The scintillation mixture consisted of a 2 to 1 mixture of toluene and methyl cellosolve containing 5 grams of PPO per liter. All fluid samples and extracts were assayed directly by counting aliquots of 0.2 to 0.5 ml. Total radioactivity in blood, tissues, feces, and substrate solids after extraction was determined by oxygen combustion in a Parr double-valved bomb. Approximately 1 gram of whole blood was placed in a small bag made from cellulose dialysis tubing (Kelly et al., 1961) and dried overnight at 40°C. The dried sample was combusted in oxygen at 25 atm. pressure, and the resulting carbon-14 dioxide was trapped in 20 ml. of a mixture of 2 to 1 methyl cellosolve and monoethanolamine (Jeffay and Alvarez, 1961). A 2-ml aliquot of the trap solution was radioassayed by liquid scintillation counting.

For determination of blood cholinesterase levels, freshly drawn heparinized blood was centrifuged to separate the RBC's from the plasma. After the plasma was decanted, the RBC's were diluted to the original blood volume with distilled water. They were not washed prior to dilution. Cholinesterase activity in the plasma and RBC's was measured using a radioisotopic method (Reed et al., 1966).

Extraction - Radioactive residues were extracted from the milk using the basic procedure of Timmerman et al. (1961). Briefly, the method called for the thorough mixing of whole milk, 50 ml., with acetonitrile to precipitate the milk solids, and then for the addition of chloroform so that an aqueous and an organic solvent layer was obtained. With the original method, the residues in the two liquid phases and in the milk solids would be quantitated at this point. In the present study, the aqueous phase was further concentrated to approximately 10 ml. and reextracted in a manner identical to the whole milk. This allowed more complete removal of the solids suspended in the aqueous portion of the milk and resulted in greater extraction of radioactive residues into the organic solvent fraction. For future reference, residues in the organic solvent fraction will be referred to as organo-extractables, those in the water layer as water-solubles, and radioactive residues remaining in the milk solids as unextractables.

Urine was extracted with chloroform, and the organo-extractable metabolites were characterized using the same techniques described for evaluating the nature of the organo-extractables from milk. Partial cleanup of the water-soluble metabolites from urine was accomplished on a Sephadex column. Sephadex LH-20, after being placed in distilled water for 3 hours and then in acetone for 10 minutes, was added to a chromatographic column 2.5 cm. in diameter, until a column bed of 10 cm. was attained. The column was then washed with 50 ml. of acetone. Five milliliters of the chloroform-extracted urine was concentrated to approximately 0.2 ml. and transferred to the column. One hundred milliliters of acetone was passed through the column to elute any organo-extractable metabolites remaining in the urine after extraction with chloroform. This was followed by 100 ml. of a 6 to 1 mixture of acetone and methanol and finally by 100 ml. of a 1 to 1 mixture of acetone and methanol. The 6 to 1 solvent system served to remove certain interfering materials. Those radioactive materials eluted with the 1 to 1 solvent mixture were considered as the true-water-soluble metabolites.

The liver was the only tissue in which attempts were made to extract the radioactive products. Acetone, benzene, n-butanol, ethanol, methanol, water, and mixtures thereof were used in efforts to extract the residues.

Thin Layer Chromatography - Techniques used to separate and identify the radioactive organo-extractable metabolites were the same as reported earlier (Andrawes et al., 1967; Dorough and Ivie, 1968). Metabolite designations are those used by Dorough and Ivie (1968).

Their separation by thin layer chromatography is shown in Table 1.

After elution from the column, the water-soluble metabolites from urine were resolved by thin-layer chromatography (TLC) using plates prepared from Silica Gel G slurried in a 0.1M boric acid solution. The chromatograms were developed in a 5 to 4 to 1 mixture of acetone, n-butanol, and 0.1M boric acid. After the radioactive areas on the plates were located by radioautography, they were extracted from the gel with methanol.

Analysis of Water-Soluble Metabolites - Attempts were made to cleave the aglycones from the urine water-soluble metabolites by acid and enzymatic hydrolysis. For acid hydrolysis, each metabolite(s) extracted from the silica gel was incubated in 2N HCl for 30 minutes at 95°C. Similarly, each metabolite(s) was incubated in various enzyme preparations at 37°C for as long as 3 days. Enzymes (Sigma Chemical Co.) used in these studies and the pH of the incubation media were as follows: beta-glucuronidase Type H-1, pH 5.0; beta-glucuronidase Type 1, pH 6.9; beta-glucosidase, pH 5.3; sulfatase, pH 5.0; protease, pH 7.5; maltase, pH, 6.4; and alpha-amylase, pH 6.9.

Following incubation, the mixtures were extracted with chloroform and the percentage conversion of water-solubles into organo-extractables was determined. The radioactive components of the chloroform extract were separated by TLC and their chromatographic behavior was compared with Aldicarb metabolite standards and with the original organo-extractable metabolites from the urine.

Results and Discussion

Effect on Animals - The pilot study indicated that feeding a dairy cow a diet containing 1.2 p.p.m. Aldicarb equivalents would not cause any visible effects to the animal. Moreover, the blood cholinesterase levels were not reduced and the quantity of milk produced by the cow remained constant. Hay and grain consumption, 27 pounds per day, and milk production, 30 pounds per day, were well below that for the other animals used in this study (Table 2). However, these values were considered normal for this particular cow since they were the same as the pretreatment figures. The fact that this cow was in the latter stage of lactation and was isolated from other animals during the feeding experiment could account for the low feed consumption and milk production. Urine and feces from the pilot cow were not quantitated.

Even though the pilot cow did not consume 50 pounds of feed per day, the animal was administered that amount of Aldicarb and Aldicarb sulfone which would be present in 50 pounds of feed containing 1.2 p.p.m. of Aldicarb equivalents. Since this dose of insecticide was not harmful to the animal, the Aldicarb feeding study involving three cows was initiated.

As observed in the pilot study, there were no apparent harmful effects to the cows resulting from Aldicarb in the diet at 0.12 p.p.m., 0.6 p.p.m., or 1.2 p.p.m. Blood cholinesterase levels were the same during the time Aldicarb was being fed as they were before treatment commenced. Milk production, feed consumption, and quantity of excretory products remained stable throughout the experiment. Each animal consumed approximately 47 pounds of feed per day but varied slightly in the amount of milk produced (Table 2). However, there was little day-to-day variation in the amount of milk produced by an individual animal.

Elimination of total Aldicarb Equivalents - The amount of the administered radioactive Aldicarb eliminated from the cows in the milk, urine and feces is shown in Table 3. Although all samples collected were analyzed, only those data sufficient to demonstrate the pattern of elimination are presented. The quantity of radioactivity eliminated by these three routes stabilized very rapidly. After 3 days, the concentration of

Aldicarb-C^{14} equivalents in the milk, urine, and feces was very close to the maximum detected at any time during the study. The fact that there was a consistent relationship between the amount of Aldicarb consumed and the rate of elimination of residues from the body was apparent from the level of radioactivity detected in the blood. After 2 days on the Aldicarb diet, residues of Aldicarb-C^{14} equivalents in the blood of the cow fed 0.12 p.p.m. were maintained at approximately 3 p.p.b. The blood of animals fed Aldicarb at 0.6 and 1.2 p.p.m. contained approximately 8 and 18 p.p.b., respectively, after the second day of feeding.

Most of the consumed Aldicarb-C^{14} was eliminated from the cows in the urine (Table 3). Regardless of the level of treatment, about 90% of the daily dose could be accounted for in urine collected the following day. There was a slight, but continuous, increase in the percentage of the dose eliminated in the urine during the feeding of radioactive Aldicarb. This may have resulted from an increase in efficiency of elimination of the daily doses by the animals or from the release of products stored from the earlier feedings. The same type of increase was noted in the feces. Whereas from 1 to 2% of the dose was voided in the feces after the first day, 3 to 3.5% of the cumulated C^{14}-treatments was eliminated in the feces by the 14th day. By adding the percentage of the dose eliminated in the milk, approximately 1.0%, to the corresponding values for urine and feces, it was concluded that dairy animals on a continuous diet containing Aldicarb would consistently eliminate 90% or more of the insecticide consumed daily.

Milk - Parts per billion of Aldicarb-C^{14} equivalents in the milk were directly related to the concentration of Aldicarb in the diet, Table 4. The level of C^{14}-residues in milk from the cow fed 0.12 p.p.m. of Aldicarb ranged from 0.9 to 1.9 p.p.b. and averaged 1.4 p.p.b. For the 0.6 p.p.m. feeding level, the residues in the milk ranged from 5.0 to 6.5 p.p.b. and at the 1.2 p.p.m. level, the residues ranged from 12.1

to 15.3 p.p.b. The average concentrations of Aldicarb-C^{14} equivalents in milk resulting from the latter two feeding levels were 5.7 and 13.3 p.p.b., respectively. The ranges cited above do not include values obtained from samples collected within 12 hours of the first treatment. Radiolabeled residues had not reached the point of stabilization at that time and did not represent a true continuous feeding situation. The average values include data from 28 separate milk samples for each feeding level, two samples each day for 14 days.

The nature of the radioactive residues in the milk is shown in Table 4. Again, analyses were performed on each milk sample collected throughout the feeding study, but only selected data are presented because the results were almost identical for all samples within a given treatment. As demonstrated in Table 4, the relative concentration of metabolites in the milk remained fairly constant during the 14-day feeding period. These results indicated that the metabolism of Aldicarb was not altered by continuous feeding of the insecticide or by increasing its concentration in the diet from 0.12 to 1.2 p.p.m.

Oximes and nitriles made up approximately 50% of the radiolabeled metabolites. Nitrile sulfone was the major metabolite, accounting for approximately 60% of the known hydrolytic products and 30% of all radioactive residues in the milk.

Aldicarb sulfone was the principal carbamate in the milk, constituting from 15 to 19% of the radioactive products present. Aldicarb sulfoxide was present at about one-fourth this level. Their combined concentrations were 0.3, 1.0, and 2.7 p.p.b. in milk from cows fed Aldicarb at the three dosage levels. The parent compound, Aldicarb, was not detected in any of the milk samples.

Collectively, the organo-extractable metabolites listed as unknowns in Table 4 composed 15% of the total residues. However, the maximum concentration of these unknown metabolites, even in milk of the cow fed the high level of Aldicarb, was only 2.2 p.p.b.

After concentrating the aqueous fraction of the milk extract and thoroughly re-extracting it with acetonitrile and chloroform, there were no detectable water-soluble metabolites present in the milk (Table 4). Without this additional extraction, however, from 10 to 15% of the radioactivity in the milk remained in the water phase. Independent analysis of these water-soluble products revealed that approximately 65% were bound with the milk solids, 15% as Unknown 3, 10% as Aldicarb sulfoxide, 5% as Aldicarb sulfone, and 5% as Unknown 3a.

Radioactivity remaining with the milk solids after extraction accounted for 15 to 20% of the total labeled-residues in the milk. Although sizable when considered in relation to their percentage of the total residues, their absolute concentration was very low. These values were 0.3, 0.9 and 2.1 p.p.b. for the three treatment rates, respectively (Table 4). No attempt was made to characterize the radioactive metabolites of Aldicarb located in the solid fraction of the milk.

Urine - Radioactive metabolites of Aldicarb in the urine increased proportionally with the increased levels of Aldicarb in the diet of the cows (Table 5). As was the case in the milk, however, the relative concentrations of the metabolites were strikingly similar regardless of the levels of Aldicarb fed.

Only about 25% of the radiolabeled products in the urine was extractable with chloroform. Of these, approximately 40% was identified as Aldicarb sulfoxide and Aldicarb sulfone, 50% as oximes and nitriles, and 10% was unknown materials. At the low feeding level, the average concentrations of these products were 11, 14 and 3 p.p.m., respectively. Urine from cows fed 0.6 and 1.2 p.p.m. contained residues corresponding to the increased dosage rates. With the exception of Unknown 5, found only in the milk, the organo-extractable metabolites in the milk and urine were the same.

Seventy-five per cent of the radioactivity in the urine remained in the aqueous phase after extraction with chloroform (Table 5) and was resolved by TLC into four distinct radioactive bands (Table 6). When unextracted urine was concentrated and applied to the chromatograms, two additional bands were observed, one with a R_f value of 0.60, band 5, and the other a R_f of 0.76, band 6. Examination of these bands individually on TLC, using the solvent systems for organo-extractables, showed that the R_f 0.6 material cochromatographed with Aldicarb sulfoxide and that the R_f 0.76 band was a mixture of other organo-extractable metabolites. In this same solvent system, the radioactive materials of bands 1 through 4 (Table 6) stayed at the origin.

TLC analysis of chloroform-extracted urine disclosed that small amounts of bands 5 and 6 were still present, demonstrating that the partitioning characteristics of the organo-soluble metabolites make it virtually impossible to attain complete extraction. Although their combined concentration was only 1 to 3% of the radioactivity in the extracted urine, it was sufficient to interfere with subsequent analysis of the water-soluble metabolites. This problem was solved by removing the organo-extractable radioactivity from the urine by Sephadex column chromatography. That material eluted from the column with a 1 to 1 mixture of acetone and methanol contained only the four radioactive bands shown in Table 6. The organo-extractable materials were eluted beforehand with acetone. In addition to allowing complete separation of the two classes of metabolites, the Sephadex column removed much of the interfering material from the radioactivity and transferred the water-soluble metabolites to an organic solvent. The latter could be more readily reduced in volume for application to TLC and gave improved separation of metabolites.

Water-Soluble Metabolites - Cleavage of water-soluble metabolites of

Aldicarb from urine by enzymatic means was almost totally unsuccessful. The maximum conversion of water-soluble metabolites to organo-extractable materials came when the incubation mixture consisted of beta-glucuronidase Type H-1 in pH 5 buffer. After 3 days at 37°C, 7% conversion had taken place. Increasing the amount of enzyme and/or the period of incubation failed to increase the quantity of organo-extractable materials. Two-dimensional TLC chromatography (Dorough and Ivie, 1968) of the aglycones showed the presence of three products, one chromatographing with Unknown 3 of the original organo extractables, one chromatographing with Unknown 3a, and the other chromatographing with oxime sulfone. Exact quantitation and identification were not possible because of the small quantity of radioactivity.

Acid hydrolysis of the water-soluble metabolites yielded much higher amounts of organo-solubles than did the enzymatic method. For these studies, radioactive bands 1 through 4 (Table 6) were incubated separately in 2N HCl at 95°C for 30 minutes. Each of the bands yielded several organo-extractable products after being resolved by TLC. However, there was a single major product produced in every case. With band 1, 72% of the water-solubles was cleaved by the acid, and 95% of these were in the form of an unknown which was designated as Unknown A. Band 2 was hydrolyzed 80% by the acid and 96% of the aglycones was as a material designated Unknown B. Acid hydrolysis of band 3 gave only 45% cleavage of the water-soluble materials while 73% of band 4 was cleaved. Both band 3 and 4 aglycones were in the form of Unknown B in excess of 95%.

Unknowns A and B were products which did not cochromatograph with any of the metabolite standards of Aldicarb. Thus, the watersolubles in the urine were not conjugates of the free Aldicarb metabolites or the free metabolites, if formed by acid hydrolysis, were unstable in the incubation medium. Incubating the Aldicarb metabolite standards in acid and then examining the products extracted with chloroform showed that Aldicarb and the standards were highly unstable under these conditions.

This made it impossible to identify the aglycones as they existed as part of the water-soluble metabolites.

Since the enzymatic cleavage of the water-soluble metabolites failed and the acid cleavage resulted in the destruction of the aglycones, only indirect evidence is available concerning the identity of the Aldicarb-contributing portion of the conjugate metabolites. When Unknown A and Unknown B, formed by acid hydrolysis of the conjugates, were compared with the unknowns produced from the Aldicarb standards upon acid hydrolysis, certain products were identical chromatographically. The major acid degradation product of oxime sulfone cochromatographed with Unknown B. That oxime sulfone was the only standard yielding Unknown B suggested that it could be the major component of the water-soluble metabolites of Aldicarb in the urine. In fact, over 95% of the radioactivity in bands 2 through 4 (Table 6) was degraded to the same product by acid hydrolysis as was oxime sulfone. Only band 1, which remained at the origin of the TLC, was degraded to a large extent to Unknown A. This unknown was not the principal degradation product of any of the Aldicarb standards when placed in the acid hydrolysis conditions. However, it did cochromatograph with Unknown 3 of the organo-extractable metabolites. It is possible, therefore, that approximately 5% of the urine water-solubles was conjugates of an unknown aglycone and that which remained was conjugates of oxime sulfone. While positive identification of the conjugate metabolites of Aldicarb must await further evaluation, these data offer strong evidence that Aldicarb, Aldicarb sulfoxide, and Aldicarb sulfone, which are of obvious toxicological importance, do not directly contribute to their formation.

Tissues - Of 27 different tissue samples analyzed for total Aldicarb-C^{14} equivalents, detectable residues were observed in 22 tissues from the cow fed 1.2 p.p.m. Aldicarb in the diet, in 20 tissues from the cow fed 0.6 p.p.m. Aldicarb, and 1 sample taken from the cow fed 0.12 p.p.m. Aldicarb (Table 7). Even at the high feeding level, residues were absent in muscle tissue, fat, and bone. With the exception of those in the liver, all of the residues in the tissues from the cow fed 0.6 p.p.m. Aldicarb were considered as trace quantities since they were present at levels only slightly above the limit of sensitivity of the analytical method. Generally, the same was true for tissues from the cow fed the high level of Aldicarb. Only the liver and lungs contained residues in excess of fourfold the 4 p.p.b. limit of sensitivity. In this animal, the lungs contained 35 p.p.b. Aldicarb-C^{14} equivalents and the liver 164 p.p.b. Aldicarb-C^{14} equivalents.

The liver from the animal fed the highest concentration of Aldicarb was the only tissue in which the radioactive content was sufficient to warrant extraction and characterization of the residues. However, attempts to extract the radioactive residues failed, and nothing of their nature was determined. The fact that they resisted extraction so successfully might suggest that the residues in the liver were not Aldicarb-like at all but were naturally occurring products containing a mere fragment of the Aldicarb molecule. It is unlikely that products other than these would remain with the solid liver residue rather than being in the organic solvent or water phase after extraction with water, acetone, methanol, n-butanol, or hot ethanol as was found to be the case.

Analytical Considerations - The present study showed that the parts per million level of total residues in the milk of cows fed Aldicarb were approximately 1/100 that level in the diet (Table 4). This relationship held true for feeding levels varying from 0.12 to 1.2 p.p.m., and in animals where the average milk production varied from 41 to 58 pounds per day. Because of this, one should be able to predict with a high degree of accuracy the concentration of residues in milk when the level of Aldicarb in the diet is known. However, the actual quantitation of Aldicarb equivalents in milk may be difficult.

Since the residues in milk of cows fed Aldicarb-contaminated feed were so low, it is unlikely that they would be detected by conventional analytical methods. Even on a total Aldicarb-equivalent basis, a method would have to be sensitive below the 0.01-p.p.m. level to detect residues in milk of animals fed 1 p.p.m. Aldicarb in the diet. To detect only the known carbamate materials, Aldicarb sulfoxide and Aldicarb sulfone, the sensitivity of the method would have to be greater than 0.002 p.p.m. Lower feeding levels of Aldicarb would obviously demand even greater sensitivity if residues were to be quantitated.

For monitoring purposes and for certain investigational uses, it would be possible to use the urine as an indicator of residue levels in the milk. The p.p.m. Aldicarb equivalents in urine (Table 5) were approximately 100 times greater than those in the milk, and the same as the p.p.m. Aldicarb fed in the diet. It would be possible to detect combined Aldicarb sulfoxide and Aldicarb sulfone in urine if cows were fed Aldicarb in the diet at concentrations as low as 0.12 p.p.m. This would require a sensitivity of only 0.01 p.p.m. If the two compounds were detected, it would indicate that their combined concentration in the milk (Table 4) was about one-fiftieth that observed in the urine. If total residues were detected, the indicated residues in the milk would be 1/100 that in the urine.

Comparison of Single-Dose and Continuous-Feeding Studies - Generally, there was good agreement between results reported by Dorough and Ivie (1968) and those obtained in the current tests. In the earlier study, Aldicarb-S^{35} was given as a single oral dose at a rate equivalent to approximately 3.5 p.p.m. in the diet. During the first 24 hours after treatment, 83% of the dose was eliminated in the urine and 1% in the milk. These values are very close to that amount eliminated 24 hours after the first Aldicarb-Aldicarb sulfone doses (Table 3). The feces of the Aldicarb-S^{35} treated cow contained a lower percentage of the dose after 24 hours than did the Aldicarb-Aldicarb sulfone treated animals, 0.6% as compared with about 2%. However, the total eliminated by this route after the single treatment, 2.9% of the dose, was almost identical to the average daily values observed in the continuous feeding study.

The similarities between the results of the single- and continuous-Aldicarb feeding studies were maintained to a large degree when the residues in milk were considered in detail. The average concentration of Aldicarb equivalents in milk collected during the first 24 hours after the Aldicarb-S^{35} treatment was 39 p.p.b., three times the 14-day average value in milk from the cow treated with Aldicarb plus Aldicarb sulfone at 1.2 p.p.m. The threefold increase was coincident with the higher treatment rate, 3.5 p.p.m. Aldicarb in the diet, used in the single treatment study.

There were two differences noted when the chemical nature of residues in the milk from the single- and continuous-Aldicarb feeding studies were compared. First, the 24-hour milk from the Aldicarb-S^{35} study had 67% of the radioactivity in the organo-extractables, 23% in the aqueous milk phase, and 10% in the milk solids. Corresponding values for the Aldicarb-Aldicarb sulfone feedings were approximately 80, 0, and 20%. These differences may be explained by incomplete extraction and separation of the milk phases in the initial study. As pointed out earlier, improved extraction techniques were utilized in this experiment.

The second difference in the chemical nature of residues in milk from the two studies was the presence of metabolite Unknown 3a in the latter study (Table 4). This material was not detected in the Aldicarb-S^{35} test but was evident in milk from all three animals treated with Aldicarb-Aldicarb sulfone-C^{14} for 14 days. It also was detected in the urine of these cows. The fact that Unknown 3a was detected in samples of milk and urine collected within 1 day after treatment began shows that the metabolite did not result from continuous treatment. Therefore, the metabolite must be an initial metabolic product of Aldicarb and/or Aldicarb sulfone. Recent studies in our laboratory on the metabolism of Aldicarb in chickens showed that metabolite Unknown 3a was present in milk and urine of the cow treated with a single dose of Aldicarb-S^{35} and Aldicarb-S-methyl-C^{14}. These data indicate that Unknown 3a was present in milk and urine of the cow treated with a single dose of Aldicarb-S^{35}. Possibly, its presence was not detected because of the frequent intervals (3, 6, 12, and 24 hours after treatment) in which the samples were collected. Sampling in this manner could prevent the accumulation of a relatively slowly-formed metabolite to a detectable level.

With only minor exception, then, it is apparent that the continuous exposure of dairy animals to Aldicarb in the diet does not significantly alter its fate as compared to a single exposure. Therefore, long-term feeding studies to determine the relationship of levels in the diet to residues in animal products when dealing with rapidly metabolized and rapidly excreted compounds such as Aldicarb are not required. Also, it is evident that a single-dose study, usually designed to determine the general metabolic fate of a compound, can be very useful in estimating the concentration and nature of residues which might occur in consumable products of animals receiving insecticides of this type in the diet.

TABLE 1. DESIGNATIONS USED FOR ALDICARB AND ITS METABOLITES AND THEIR SEPARATION BY TLC

	R_f Values[b]	
Designation[a]	1	2
Aldicarb-C^{14}	0.67	0.94
Aldicarb sulfoxide	0.05	0.18
Aldicarb sulfone-C^{14}	0.21	0.64
Aldicarb oxime	0.78	0.95
Oxime sulfoxide	0.18	0.22
Oxime sulfone	0.56	0.78
Nitrile sulfoxide	0.31	0.70
Nitrile sulfone	0.62	0.93
Unknown 1	0.05	0.11
Unknown 2	0.10	0.24
Unknown 3	0.11	0.34
Unknown 3a	0.36	0.41
Unknown 5	0.47	0.86

[a] Metabolite designations from Dorough and Ivie (1968)

[b] Two dimensional TLC. First solvent system, 2:1 ether-hexane + 20% acetone and second system, 2:1 methylene chloride-acetonitrile.

TABLE 2. TREATMENT RATES AND FEED CONSUMPTION FOR DAIRY COWS FED ALDICARB FOR 24 DAYS[a]

	Average per day values/cow no.			
	Pilot	1	2	3
Aldicarb equivalents in feed, p.p.m.	1.2	0.12	0.6	1.2
Aldicarb equivalents, mg/kg (body wt)	0.042	0.006	0.027	0.052
Feed consumption, lbs.	27	46	48	47
Milk production, lbs.	30	41	50	58

[a] The pilot cow was given nonradioactive aldicarb for 10 days while the other animals received the insecticide for a total of 24 days, 10 days on nonradioactive material and 14 days on radiolabeled products.

TABLE 3. ELIMINATION OF RADIOACTIVITY BY COWS FED ALDICARB-C^{14} FOR 14 DAYS AT RATES OF 0.12, 0.6 AND 1.2 P.P.M. IN THE DIET

	Percent of total dose for each treatment level in[a]								
Days fed	Milk, p.p.m.			Urine, p.p.m.			Feces, p.p.m.		
insecticide	0.12	0.6	1.2	0.12	0.6	1.2	0.12	0.6	1.2
1/2	0.9	0.5	0.7	68.7	75.6	74.4	1.3	0.5	0.8
1	0.8	0.7	1.1	82.0	81.9	83.6	2.0	1.1	1.6
2	0.7	0.8	1.2	86.0	85.1	85.8	3.1	2.0	1.9
3	0.7	0.8	1.2	90.1	89.7	89.7	3.3	2.4	2.3
7	0.8	0.9	1.3	90.7	88.5	90.8	3.4	2.5	2.6
10	0.8	0.9	1.3	90.9	90.5	90.4	3.5	2.8	2.8
12	0.9	0.9	1.3	93.1	91.2	91.0	3.5	2.9	2.8
14	0.9	0.9	1.3	93.8	91.6	92.1	3.5	3.0	2.9

[a] Calculations based on total dose consumed and total radioactivity eliminated by each indicated time.

TABLE 4. RADIOACTIVE COMPONENTS IN MILK OF COWS FED ALDICARB-C^{14} AT RATES OF 0.12, 0.6, AND 1.2 P.P.M. FOR 14 DAYS

	P.P.B./14-day average		
	Cow #1 (0.12)	Cow #2 (0.6)	Cow #3 (1.2)
Metabolites	14-day avg.	14-day avg.	14-day avg.
Organo-extractables[a]			
Aldicarb sulfoxide	0.06	0.22	0.40
Aldicarb sulfone	0.21	0.85	2.24
Oxime sulfoxide	0.05	0.27	1.13
Oxime sulfone	0.07	0.34	0.94
Nitrile sulfoxide	0.05	0.17	0.61
Nitrile sulfone	0.44	2.22	3.71
Unknown 1	0.01	0.04	0.08
Unknown 2	0.04	0.12	0.27
Unknown 3	0.08	0.29	0.74
Unknown 3a	0.08	0.22	0.96
Unknown 5	0.02	0.06	0.11
Water-solubles	0	0	0
Milk solids	0.28	0.91	2.06
Total	1.39	5.71	13.25

[a] Metabolites extracted from whole milk with acetonitrile and chloroform.

TABLE 5. RADIOACTIVE COMPONENTS IN URINE OF COWS FED ALDICARB-C^{14} AT RATES OF 0.12, 0.6, AND 1.2 P.P.M. FOR 14 DAYS

	P.P.B./14-day average		
	Cow #1 (0.12)	Cow #2 (0.6)	Cow #3 (1.2)
Metabolite	14-day avg.	14-day avg.	14-day avg.
Organo-extractables[a]			
Aldicarb sulfoxide	4.4	25.6	43.6
Aldicarb sulfone	7.3	35.9	66.5
Oxime sulfoxide	7.7	34.7	96.4
Oxime sulfone	4.9	32.3	43.6
Nitrile sulfoxide	1.0	3.6	8.0
Nitrile sulfone	1.5	6.7	10.3
Unknown 1	0.1	0.6	1.1
Unknown 2	0.6	2.4	6.8
Unknown 3	1.1	7.9	8.0
Unknown 3a	1.2	3.6	16.0
Water-solubles	96.0	465.2	847.2
Total	125.8	609.5	1147.5

[a] Metabolites extracted from urine with chloroform.

TABLE 6. THIN LAYER SEPARATION[a] OF RADIOACTIVE WATER-SOLUBLE METABOLITES IN URINE

	Percent of water solubles/days				
Radioactive band	R_f	3	7	10	14
1	0	5.1	5.8	3.1	5.0
2	0.1	42.2	40.4	46.7	38.9
3	0.17	42.8	46.4	42.0	40.9
4	0.39	9.9	7.4	8.2	15.2

[a] TLC plates prepared with Silica Gel G in 0.1M boric acid. Solvent system consisted of a 5:4:1 mixture of acetone, n-butanol, and 0.1M boric acid.

TABLE 7. RADIOLABELED RESIDUES IN TISSUES OF COWS FED ALDICARB-C^{14} FOR 14 DAYS[a]

	P.P.B. aldicarb equivalents at indicated feeding level[c]		
Tissues[b]	Cow #1 (0.12 p.p.m.)	Cow #2 (0.6 p.p.m.)	Cow #3 (1.2 p.p.m.)
Liver	29	123	164
Lungs	-	7	35
Kidney	-	6	16
Bile	-	9	16
Adrenal glands	-	6	12
Abomasum	-	4	11
Omasum	-	4	11
Large intestine	-	5	10
Ovaries	-	6	10
Rumen	-	5	10
Udder	-	6	10
Pancreas	-	5	9
Spinal cord	-	-	9
Gall bladder	-	4	8
Heart	-	6	8
Reticulum	-	6	8
Spleen	-	5	8
Skin	-	4	7
Small intestine	-	5	7
Brain	-	4	6
Neck muscle	-	-	6
Tongue	-	4	6

[a] Animals slaughtered 18 hours after last treatment.

[b] Residues were not detected (below 4 p.p.b.) in the following tissues: foreleg muscle, hindleg muscle, omental fat, subcutaneous fat, and rib bone.

[c] - indicates residues below 4 p.p.b.

Aldicarb in Laying Hens

Recent reviews of aldicarb metabolism (Dorough, 1970; Kuhr, 1970) pointed out that sulfur oxidation and hydrolysis of the carbamate ester were of major importance in the metabolism of this carbamate. Subsequent work supported this view and has extended the study of aldicarb metabolism to include the synthesis and identification of highly polar products not considered previously (Bartley et al., 1970; Durden et al., 1970). The metabolic pathway for aldicarb presented by Bartley et al. (1970) is the most complete of any reported thus far. It is likely that this pathway is representative of the type of metabolism which aldicarb undergoes in the other biological systems tested.

It was the intent of the current study to determine if laying hens metabolized aldicarb in the same manner as reported for other organisms. In addition, these investigations were designed to provide evidence for the levels and chemical nature in residues in eggs and tissues of hens which consume aldicarb residues in the diet. The sulfone analog was included since it is a common metabolite in plants which may serve as poultry feed (Bartley et al., 1970).

Methods

Insecticides - Aldicarb-S-methyl-^{14}C and aldicarb sulfone-S-methyl-^{14}C each with a specific activity of 5 mCi/mmol, and aldicarb-S^{35}, 45.7 mCi/mmol, were supplied by the Union Carbide Corp., as were a series of metabolite standards. The radioactive materials contained less than 1% radioactive impurities as determined by thin-layer chromatography (tlc) and radioautography.

Treatment and Sampling - In the first experiment, ten White Leghorn laying hens were treated with a single oral dose of aldicarb-S^{35} at a rate of 0.7 mg/kg. The birds, each weighing approximately 1.5 kg were administered the radioactive aldicarb via a 5 grain gelatin capsule which contained a small amount of laying mash. Two hens were sacrificed at 6 hr, and 1, 3, 5, and 10 days after treatment. Eggs and feces were collected at 6-hr intervals during the first 12 hr and then at 24-hr intervals thereafter. In this and all other tests described herein, the birds were maintained in air-conditioned housing under continuous lighting. Water and laying mash were provided ad libitum.

In another test, six laying hens received a 1:1 molar ratio, single oral dose of aldicarb-^{14}C and aldicarb sulfone-^{14}C. Doses of the insecticides were prepared in gelatin capsules so that each hen received 0.7 mg/kg of aldicarb equivalents. The birds were sacrificed 6 hr, 1 day, and 3 days after the carbamates were administered. Eggs and feces were collected as described above.

To study the fate of aldicarb residues when consumed by hens for an extended period, aldicarb-^{14}C and aldicarb sulfone-^{14}C, 1:1 molar ratio doses, were administered to hens every 12 hr for 21 days. Based on an average feed consumption of 80 g per bird per day, the treatment levels corresponded to aldicarb levels in the diet of 0.1, 1.0 and 20.0 ppm. Six hens were used for each treatment level, and another six birds served as control animals. Nonradioactive insecticides were given to the birds for 7 days prior to initiating the radioactive feeding. Each capsule contained half the total amount of aldicarb equivalents required in 1 day's ration to obtain the desired ppm level in the diet. Eggs and feces were collected twice daily just prior to administering the radioactive aldicarb and aldicarb sulfone. Three hens from each feeding level were sacrificed 12 hr after the last treatment and the remaining three hens were killed on the seventh day following the last treatment. Tissue samples were collected and frozen until analyzed.

Radioassay - Radioactive measurements were accomplished on a Packard Tri-Carb Model 3380/544 liquid scintillation counter. The scintillation mixture and the details of counting liquid and solid samples were the same as described in a similar study with carbofuran (Hicks et al., 1970). The blood was radioassayed by oxygen combustion techniques, as were all solids, after 1 g of blood was evaporated to dryness in a bag made from dialysis tubing (Andrawes et al., 1967).

Extraction of Residues - Feces, 20 g, were extracted by blending with 40 ml of water, followed by the addition of 150 ml of acetonitrile with continued blending for about 1 min. The homogenate was filtered and the feces solids were extracted with 60 ml of a 2:1 mixture of acetonitrile and water. The combined homogenates were washed five times with 80 ml portions of hexane which was discarded because the hexane contained only negligible amounts of radioactivity. The acetonitrile-water phase was extracted thoroughly with chloroform and the aqueous and organic solvent layers were radioassayed. The latter phase was decolorized with a small quantity of activated carbon, filtered, and concentrated for spotting on tlc.

Egg whites, 40 g, were extracted three times; each time the homogenates were filtered and the solids returned to the blender for further homogenization. The first solvent was 60 ml of acetonitrile, the second was 90 ml of a 2:1 mixture of acetonitrile and water, and the third was 60 ml of hexane. All extracts were combined, shaken, and the layers allowed to separate completely. The acetonitrile-water extract was removed and washed twice with 40-ml portions of hexane. Chloroform was added to separate the acetonitrile and water, and the procedure continued as described above for the feces.

Egg yolks (20 g) were homogenized in 40 ml of water, 60 ml of acetonitrile were added, and homogenization continued for 3 min. The remainder of the extraction and cleanup was identical to that used for the egg whites. Equal extraction efficiency, but an acetonitrile extract with less oils, was obtained when the egg yolks were extracted, using the more involved method described below for tissues.

The various tissues were chopped into small pieces and a 40-g subsample, or all available, was analyzed for residues. The subsample was homogenized in 60 ml of a 2% potassium oxalate solution for 5 min. Ethanol (50 ml) was added to the homogenate and the mixture was homogenized for 3 min. The entire homogenate was transferred to a separatory funnel containing 100 ml of ether and 50 ml of pentane, mixed, and the water layer removed. The water was again extracted with the ether and pentane mixture. After separating the layers, 100 ml of acetone were added to the aqueous phase and shaken thoroughly. The solids, formed on shaking with acetone, were removed by filtration and 10 ml of acetone and 100 ml of ether were used to wash the solids. The filtrate was transferred to a separatory funnel, shaken, and the layers were separated. All organic solvent extracts were combined, dried with anhydrous sodium sulfate, and concentrated to an oily residue.

The residue was transferred to a separatory funnel with 30 ml of hexane and 30 ml of acetonitrile. The funnel was shaken, phases were separated, and the hexane was extracted twice more with acetonitrile. The combined acetonitrile extracts were washed with 20 ml of hexane. Following this final wash to remove the oils, the acetonitrile extract was prepared for tlc analysis.

Thin-Layer Chromatography - Because tlc was used to separate the metabolites of aldicarb, and cochromatography of the unknown products with authentic standards was the major means of metabolite identification, great care was taken in developing several efficient tlc systems. The supports consisted of silica gel plates coated 0.3 mm thick and Chromar 500 thin-layer sheets (Mallinckrodt, St. Louis, Mo.). A series of two-dimensional solvent systems was utilized in achieving complete separation of metabolites and for establishing cochromatography in multiple solvent systems. The basic system was a 2:1 ether-hexane + 20%

acetone for the first direction and a 2:1 methylene chloride-acetonitrile mixture for the second dimension (Dorough and Ivie, 1968). Organic extracts of the various substrates were spotted on the tlc along with a mixture of metabolite standards. Following development of the tlc, the plates, or sheets, were exposed to iodine vapors or sprayed with a 1% potassium permanganate solution to visualize the standards. Radioautography was used to locate the radioactive areas on the tlc's.

When there was an indication that one of the standards cochromatographed with an unknown metabolite, the metabolite was isolated and additional tests for cochromatography were conducted. For these experiments, various combinations of solvent systems were used to confirm or rule out the two-dimensional cochromatography of the two materials. The solvent systems were as follows: 5:1 ethyl acetate and methanol; 1:1 dioxane and hexane; 9:1 dioxane and methanol; and a 5:1:1 mixture of chloroform, ethyl acetate, and hexane.

Results and Discussion

Effect of Treatments on Hens - There were no symptoms of carbamate poisoning in any of the hens treated either with the aldicarb, per se, or with the combination of aldicarb and its sulfone analog. As shown in Table 8, the 21-day treatment of hens with aldicarb and aldicarb sulfone had no deleterious effects on the birds. When compared to the control animals, the treated birds showed no appreciable differences in body weight, food consumption, egg production, or quantity of fecal matter voided from the body.

Quantity and Nature of Aldicarb Equivalents Excreted from the Body - The pattern of excretion of the aldicarb doses was similar when hens were treated with aldicarb-S^{35} alone, or with a mixture of aldicarb-^{14}C and aldicarb sulfone-^{14}C (Table 9). In both tests, the hens eliminated approximately 80% of the dose in 2 days. Extending the collection of feces from the aldicarb-S^{35} treated animals to 10 days showed that a total of 90% of the single oral dose had been excreted from the body.

The pattern of elimination of aldicarb from the hens was not altered a great deal when the insecticide was administered over a 21-day period (Table 10). After 8 to 10 days of treatment, there was an equilibrium formed between the amount of material consumed and the amount excreted. At this point, an equivalent of 80 to 85% of each daily dose was detected in feces excreted during the following 24 hr. These values were lower at the 20-ppm feeding level, 60 to 65%, although there was no apparent reason for this discrepancy in the amount of the daily doses eliminated. Removing the source of aldicarb resulted in almost 90% of the total doses consumed being excreted from the body within 1 week. Such rapid and thorough elimination of the carbamate would likely prevent the accumulation of large quantitites of residues in the tissues

The chemical fate of aldicarb in laying hens when administered as a single dose or for an extended period of time is shown in Tables 9 and 10. Approximately half of the radioactive residues in the feces consisted of unknown water-soluble metabolites. These materials were formed very rapidly by the hens, as evidenced by the results of the analysis of feces collected 6 hr after a single dose was administered (Table 9). No attempts were made to identify these metabolites. However, it is unlikely that the carbamate moiety was intact on these materials, since conjugation and/or degradation of aldicarb to alcohols and acids, processes which yield water-soluble metabolites, would be preceded by hydrolysis of the carbamate ester (Bartley et al., 1970). The unextractable radioactive metabolites, 8 to 10% of the residues, may be similar in chemical nature to the water-soluble metabolites.

Of the metabolites which were identified, aldicarb sulfoxide was the only product detected following a single dose that also was not found in the feces of birds treated repeatedly. It was apparent from the single-dose study that the sulfoxide was formed and excreted rapidly. Only trace amounts were detected in feces collected after 6 hr. Assuming that the birds on the continuous treatments did excrete aldicarb sulfoxide in the feces shortly after dosing, it must have been transformed into another material before the feces were collected and frozen. The same may have been true for the aldicarb sulfone, since it was present in higher amounts in the 6 hr feces (Table 9) than in the feces of hens used in the continuous feeding study (Table 11).

The only other metabolite of the intact carbamate identified was the N-hydroxymethyl analog of aldicarb sulfone. This product accounted for 8 to 9% of the radioactive residues in the feces of hens fed aldicarb for 21 days. Based on its relatively low content in the hens treated with a single dose of aldicarb or aldicarb sulfone, aldicarb-hydroxymethyl-sulfone was probably formed rather slowly in the hens. It reached a maximum concentration after the hens were exposed to the insecticides for approximately 8 days.

Hydrolytic products accounted for most of the other residues in the feces of the treated hens. The sulfone forms of these metabolites were predominate, with the nitrile sulfone present in highest amounts in feces of hens treated for a total of 21 days. Nitrile sulfone was also the major identified metabolite in feces of hens given a single oral dose of aldicarb-C^{35}. However, the oxime sulfone concentration was greater in the feces of hens given a single dose of aldicarb-^{14}C and aldicarb sulfone-^{14}C. This was the only marked difference noted in the quantity of individual metabolites in feces of hens receiving the two single treatments.

The two unknown metabolites, designated Unknown 4 and 5 based on earlier studies (Dorough and Ivie, 1968) were present in the feces of all tested hens. Unknown 5 was always present in greater quantities than Unknown 4, and in some cases accounted for 6% of the radioactive residues in the feces. Neither Unknown 4 or 5 cochromatographaed with any of the available standards or was present in sufficient quantity for more detailed evaluation of their chemical nature.

Eggs - Adding aldicarb sulfone to the aldicarb dose resulted in higher levels of residues in the eggs than when only aldicarb was given as a single oral dose (Table 12). In neither case did the total aldicarb equivalents exceed 0.2 ppm in the egg yolks or whites. However, maximum aldicarb equivalents of 0.18 ppm were observed when the two insecticides were administered together as compared to a maximum of 0.07 ppm when aldicarb was the only component. Whereas the radioactive residues in the whites had declined markedly by the third day, the residues in the yolks were similar or showed a slight increase by the third day. By 10 days, residues in eggs of hens treated with the aldicarb-S^{35} were 0.014 ppm in the yolk and 0.007 ppm in the whites.

Although these preliminary experiments were not designed to yield sufficient eggs for extensive analysis, the nature of the majority of the residues was tentatively projected (Table 13). Eggs containing the aldicarb-S^{35} residues showed the presence of a number of products not detected in the eggs of hens treated with aldicarb-^{14}C and aldicarb sulfone-^{14}C. This was because the sulfur-35 material was of a very high specific activity and the sensitivity for detecting the individual metabolites was much greater than with the carbon-14 insecticides.

The data (Table 13) show that none of the carbamate metabolites of aldicarb were at detectable levels in the egg yolks or whites. The water-soluble metabolites and nitrile sulfone were the predominant products in the eggs. Other hydrolytic metabolites were detected in the egg whites of the aldicarb-S^{35} treated birds. Although the data were not as complete as desired, these tests indicated the types of metabolites which could be expected in the eggs of hens consuming residues of aldicarb in the diet. The tests also suggested that the aldicarb sulfone contributed more to the residue content of the eggs than did aldicarb. In the yolks this increase was expressed as water-soluble metabolites, while in the whites there was a noted increase in the amount of nitrile sulfone.

Continuous exposure of aldicarb residues to hens resulted in some very interesting patterns of residue levels in the egg. At feeding levels of 1.0 and 20.0 ppm aldicarb equivalents in the diet, it was observed that the total ^{14}C residues in the yolk did not reach a plateau until after 12 to 15 days of feeding. This occurrence was not noted at the 0.1 ppm feeding level because the amount of residues in the eggs was only slightly above the level of sensitivity, which was 0.005 ppm total aldicarb-^{14}C equivalents when analyzing 1 g of sample.

Residues in the egg whites stabilized on the sixth or seventh day of treatment. In this case, the total aldicarb-^{14}C equivalents reached a plateau of approximately 0.006, 0.06 and 0.7 ppm for the three feeding levels. Although there were day-to-day variations in the quantities of residues in the egg whites, there were no significant increases or declines in the residue levels until the insecticide source was removed. Once the treatments stopped, the aldicarb-^{14}C equivalents in the egg whites dropped rapidly and by 7 days were almost nondetectable.

Unlike the residues in the egg yolks of hens treated with single doses of aldicarb, there was a portion of the radioactive materials in the egg yolks of these hens which partitioned into the hexane fraction (Table 14). This was an unexpected occurrence because the use of the hexane in the cleanup procedure was a means of removing the fats and oils from the acetonitrile extracts as had been done with the feces.

The identity of the hexane-soluble radioactivity from the yolks was not determined. Attempts to separate these products from the oils by tlc and extractions of various kinds were unsuccessful, and it may be that they were naturally occurring materials synthesized from S-methyl-^{14}C fragments from the carbamate materials. They were certainly not the typical types of aldicarb metabolites usually encountered.

Subtracting the hexane-soluble radioactivity from the total aldicarb-^{14}C equivalents in the egg yolks revealed that the pattern of accumulation of the remaining residues in the yolk was very similar to that in the egg whites. These residues reached a plateau on the sixth or seventh day of treatment and did not vary greatly until the carbamate treatments were terminated. Once the treatments were stopped, the non-hexane-soluble residues in the yolks declined as did those in egg whites.

With the exception of the increasing hexane-soluble radioactive materials, the relative concentrations of aldicarb metabolites remained fairly constant. For this reason, the nature of the residues in the eggs is present in Table 14 as averages of the data gathered on eggs laid the fifth through the 21st days of treatment. The N-hydroxymethyl analog of aldicarb sulfone was the only carbamate material identified in either the yolks or the whites and then only in eggs of hens at the 20-ppm feeding level. As was the case with the feces and eggs from the single-treatments studies, hydrolytic and unknown water-soluble metabolites accounted for most of the residues. The very low quantities of aldicarb-^{14}C equivalents in the eggs and their chemical nature suggested that small levels of aldicarb residues in the diet of laying hens would not result in toxicologically significant levels of residues in the eggs.

Tissues - The situation in regards to the levels and nature of residues in the tissues of the hens was very much like that described for the eggs. Feeding a combination of aldicarb and aldicarb sulfone appeared to cause slightly higher residues in the various tissues than did the treatment with just aldicarb (Table 12). Residues in the tissues declined sharply as the carbamates were eliminated from the body, and by 3 days the levels had fallen from a high of about 0.6 ppm aldicarb equivalents to levels generally below 0.1 ppm. By 10 days the radioactive residues were less than 0.03 in all the tissue analyzed.

Small quantities of the residues in the kidney and breast of hens killed 6 hr after treatment with a single dose of the insecticide were identified as aldicarb sulfoxide. Similarly low levels of aldicarb sulfone were detected in the liver and breast. All other detectable metabolites were hydrolytic products of known identity or unknown metabolites in the water fraction or were unextractable from the tissues.

Generally, the magnitudes and nature of residues in the tissues of hens treated 21 days with aldicarb and aldicarb sulfone were not too different than what had been observed with the single treatments (Tables 15 and 16). The liver and kidney contained the highest levels of aldicarb-^{14}C equivalents and the majority of these were hydrolytic products, mainly nitrile sulfone, and water-soluble unknowns. The only carbamate identified was a trace amount of aldicarb sulfoxide in the liver of birds sacrificed 12 hr after the last treatment.

There were considerable quantities of hexane-soluble products in the gizzard, liver, and kidney. As with the eggs, these materials were not present in hens other than those on the continuous treatments. Tissues of hens killed on the seventh day after receiving their last aldicarb treatment contained only nitrile sulfone in the acetonitrile fraction of the tissue extracts. The remainder of the residues was distributed among the hexane, water, and in the tissue solids as unextractable metabolites. As pointed out for the eggs, the data gathered by this investigation do not indicate that low levels of aldicarb residues in the diet of poultry would result in harmful levels of residues in the meat.

TABLE 8. TREATMENT RATES AND CRITERIA FOR DETERMINING THE EFFECT OF FEEDING EQUIMOLAR DOSES OF ALDICARB-^{14}C AND ALDICARB SULFONE-^{14}C TO LAYING HENS FOR 21 DAYS.

	Group numbers[a]			
	I	II	III	Control
Treatment rates				
Aldicarb equivalents in feed, ppm[b]	0.1	1.0	20.0	0
Aldicarb equivalents, mg/kg of body wt[c]	0.005	0.05	1.0	0
Body weight, kg				
0 Day, range, and average	1.3-1.5(1.4)	1.3-1.7(1.5)	1.4-1.8(1.5)	1.4-1.7(1.5)
21 Day, range, and average	1.3-1.6(1.4)	1.3-1.6(1.4)	1.3-1.7(1.5)	1.3-1.7(1.5)
Feed consumption g/hen/day, range, and average	64-112(74)	77-103(84)	66-106(82)	67-101(79)
Egg production Average/day/hen	0.70	0.50	0.58	0.63
Feces eliminated g/hen/day	108	109	118	98

[a] Six hens in each group.

[b] Based on an average daily food intake of 80 g for each bird.

[c] Based on an average body weight of 1.5 kg for each bird.

TABLE 9. NATURE OF RADIOACTIVITY IN FECES OF HENS TREATED WITH A SINGLE ORAL DOSE OF ALDICARB-S^{35} (A) OR AN EQUIMOLAR DOSE OF ALDICARB-^{14}C AND SULFONE-^{14}C (B)[a]

	Percent of total radioactivity in sample after					
	6 hr		1 day		1 day	
Metabolites	A	B	A	B	A	B
Aldicarb sulfoxide	5.1	13.3	0.0	0.6	0.0	0.0
Aldicarb sulfone	3.9	1.1	0	0	0	0
Aldicarb-NCH_2OH sulfone	1.0	1.6	0.9	5.8	2.7	5.5
Oxime sulfoxide	5.2	2.6	1.8	2.5	4.5	4.5
Oxime sulfone	2.8	14.6	4.1	10.6	5.6	9.9
Nitrile sulfoxide	2.3	4.2	2.3	1.3	2.4	2.9
Nitrile sulfone	7.2	4.2	9.2	5.4	16.3	10.7
Alcohol sulfoxide	1.8	1.4	2.0	1.2	5.9	2.8
Alcohol sulfone	1.7	1.6	1.7	3.0	7.7	4.9
Unknown 4	1.3	0.3	1.2	0	3.8	1.6
Unknown 5	5.9	1.9	3.3	2.0	0	0.9
Water-solubles	50.0	44.0	65.1	57.5	44.6	45.1
Unextractables	11 8	9.2	8.4	10.1	6.5	11.2
Cumulative % of dose excreted	50.2	48.0	74.3	76.1	84.5	79.3

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[a] Dosage rate = 0.7 mg/kg

[b] Values on day experiments terminated: A = 90% after 10 days; B = 82% after 3 days.

TABLE 10. ELIMINATION OF RADIOACTIVITY IN THE FECES OF HENS FED EQUIMOLAR DOSES OF ALDICARB-^{14}C AND ALDICARB SULFONE-^{14}C IN THE DIET FOR 21 DAYS

Days fed insecticides	Percent of consumed doses excreted in the feces[a]		
	0.1 ppm	1.0 ppm	20.0 ppm
1/2	76.0	76.2	64.7
1	75.3	69.8	62.7
3	77.5	71.9	64.9
11	87.9	88.6	69.8
19	83.3	82.7	70.1
21	84.9	82.6	71.0
21-day avg.[b]	82.2	80.3	67.5
Days after last treatment			
1	85.3	86.1	72.4
2	85.9	86.4	72.6
4	86.2	86.8	72.8
7	86.2	87.3	73.3

[a] Based on total dose consumed and total radioactivity eliminated by indicated time after first treatment.

[b] Average of all daily values over the 21-day feeding period.

TABLE 11. RADIOACTIVE RESIDUES IN FECES OF HENS FED EQUIMOLAR CONCENTRATIONS OF ALDICARB-^{14}C AND ALDICARB SULFONE-^{14}C IN THE DIET FOR 21 DAYS

	Percent of total radioactivity in feces at indicated feeding levels[a]		
Metabolites	0.1 ppm	1.0 ppm	20.0 ppm
Aldicarb sulfone	0.0	0.0	0.9
Aldicarb-NCH_2OH sulfone	9.1	8.1	8.7
Oxime sulfoxide	0.0	1.7	0.8
Oxime sulfone	4.3	8.4	4.9
Nitrile sulfoxide	0.0	1.5	1.0
Nitrile sulfone	10.7	12.0	9.6
Alcohol sulfoxide	5.1	2.4	3.8
Alcohol sulfone	8.2	9.6	11.5
Unknown 4	0.0	0.6	1.4
Unknown 5	5.8	3.7	4.9
Water-solubles	48.1	43.8	43.3
Unextractables	8.7	7.6	9.2

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[a] Values are averages of analysis of feces collected 1, 2, 4, 12, 17, and 21 days after initiating feeding of the radioactive insecticides.

TABLE 12. PPM ALDICARB EQUIVALENTS IN TISSUES AND EGGS OF HENS TREATED WITH A SINGLE ORAL DOSE OF ALDICARB-S^{35} (A) OR WITH AN EQUIMOLAR DOSE OF ALDICARB-^{14}C AND ALDICARB SULFONE-^{14}C (B)[a]

	Ppm (wet wt) aldicarb-^{14}C equivalents after						
	6 hr		1 day		3 days		10 days[b]
Tissue	A	B	A	B	A	B	A
Kidney	0.59	0.73	0.19	0.21	0.09	0.11	0.020
Liver	0.53	0.68	0.26	0.31	0.10	0.14	0.034
Heart	0.28	0.35	0.12	0.09	0.07	0.05	0.010
Gizzard	0.26	0.31	0.11	0.08	0.04	0.06	0.020
Skin	0.22	0.25	0.08	0.08	0.04	0.05	0.004
Breast	0.20	0.32	0.08	0.08	0.03	0.05	0.006
Thigh	0.20	0.29	0.08	0.08	0.04	0.04	0.008
Leg	0.20	0.27	0.08	0.07	0.04	0.04	0.005
Blood	0.18	0.28	0.11	0.07	0.06	0.04	0.014
Brain	0.18	0.27	0.08	0.09	0.04	0.04	0.008
Fat	0.08	0.06	0.04	0.07	0.03	0.05	0.004
Egg yolk	c	0.14	0.01	0.13	0.07	0.18	0.014
Egg white	c	0.16	0.03	0.18	0.06	0.07	0.007

[a] Dosage rate = 0.7 mg/kg.

[b] Experiment involving treatment with B was terminated after 3 days.

[c] No eggs laid.

TABLE 13. RESIDUES IN TISSUES AND EGGS OF HENS TREATED WITH A SINGLE ORAL DOSE OF ALDICARB-S^{35} (A) OR WITH AN EQUIMOLAR DOSE OF ALDICARB-^{14}C AND ALDICARB SULFONE-^{14}C (B)[a]

	Ppb in indicated tissue[b]											
	Liver		Kidney		Gizzard		Breast		Egg yolk[c]		Egg white[c]	
Metabolites	A	B	A	B	A	B	A	B	A	B	A	B
Aldicarb sulfoxide	0.0	0.0	7.7	48.9	0.0	0.0	0.0	7.4	0.0	0.0	0.0	0.0
Aldicarb sulfone	30.2	0	0	0	0	0	3.8	0	0	0	0	0
Oxime sulfoxide	8.0	14.3	9.4	0	1.3	9.9	7.0	10.8	3.2	8.2	10.6	0
Oxime sulfone	27.0	102.0	63.7	39.4	9.4	54.9	25.2	45.1	2.1	0	3.6	0
Nitrile sulfoxide	7.4	20.4	20.1	53.3	74.6	6.8	4.6	4.2	0	0	3.6	0
Nitrile sulfone	28.1	17.7	50.8	70.0	23.4	14.9	27.4	20.8	30.9	44.3	7.0	126.9
Alcohol sulfoxide	4.8	0	0	7.3	1.3	7.3	0	0	0	0	2.5	0
Alcohol sulfone	5.8	0	0	0	2.1	0	5.4	7.4	0	9.4	3.1	0
Water-solubles	353.0	439.9	373.5	379.6	105.6	182.0	83.4	160.7	28.1	57.6	24.2	29.3
Unextractables	65.7	85.7	64.9	131.5	42.4	33.8	42.8	60.0	5.7	5.7	5.5	23.8

[a] Dosage rate = 0.7 mg/kg

[b] Tissues from hens sacrificed 6 hr after treatment.

[c] A, analysis of eggs laid third day after treatment; B, eggs laid first day after treatment.

TABLE 14. RESIDUES IN EGGS OF HENS FED EQUIMOLAR CONCENTRATIONS OF ALDICARB-^{14}C AND ALDICARB SULFONE-^{14}C AT RATES OF 1.0 AND 20.0 PPM IN THE DIET FOR 21 DAYS

	Ppb at indicated feeding level[a]					
	1.0 ppm			20 ppm		
Metabolites	Yolk	White	Total	Yolk	White	Total
Acetonitrile solubles						
Aldicarb-NCH$_2$OH sulfone	0.0	0.0	0.0	0.0	4.8	3.0
Oxime sulfoxide	1.3	3.2	2.5	14.1	40.0	29.7
Oxime sulfone	1.8	1.6	1.7	4.3	21.3	13.5
Nitrile sulfoxide	0.3	0.4	0.4	42.7	27.3	33.0
Nitrile sulfone	21.1	28.5	25.7	304.5	417.4	374.5
Alcohol sulfoxide	0.0	0.2	0.1	8.3	12.0	10.1
Alcohol sulfone	0.2	0.5	0.4	11.6	16.5	14.3
Unknown 5	0.3	0.5	0.4	6.0	12.0	10.0
Hexane-solubles	52.8	0.0	17.8	495.4	0.0	189.6
Water-solubles	6.6	14.3	10.9	192.4	223.8	211.9
Unextractables	4.0	4.1	2.8	82.5	89.6	87.0
Total	88.4	53.3	62.7	1161.8	864.7	976.6

[a] Average of eggs laid 5-21 days of feeding radioactive insecticides.

TABLE 15. PPM ALDICARB-^{14}C EQUIVALENTS IN TISSUES OF HENS KILLED 12 HR (A) AND 7 DAYS (B) AFTER THE BIRDS WERE FED EQUIMOLAR CONCENTRATIONS OF ALDICARB-^{14}C AND ALDICARB SULFONE-^{14}C IN THE DIET FOR 21 DAYS

	1.0 ppm		20.0 ppm	
Tissues	A	B	A	B
Liver	0.14	0.02	1.40	0.36
Kidney	0.12	0.03	1.38	0.39
Heart	0.07	0.02	0.92	0.35
Brain	0.07	0.02	0.90	0.40
Gizzard	0.07	0.02	0.81	0.33
Blood	0.07	0.03	0.76	0.34
Leg	0.06	0.03	0.71	0.30
Skin	0.06	0.02	0.70	0.36
Thigh	0.06	0.02	0.70	0.31
Breast	0.06	0.02	0.68	0.28
Fat	0.05	0.02	0.52	0.22

[a] Residues in tissues of hens fed 0.1 ppm were below the level of sensitivity, 0.005 ppm, except in the following tissues taken 12 hr after the last treatment: blood, 0.015 ppm; kidney, 0.012 ppm; and liver, 0.011 ppm.

TABLE 16. RESIDUES IN TISSUES OF HENS AFTER A 21-DAY PERIOD OF FEEDING EQUIMOLAR CONCENTRATIONS OF ALDICARB-^{14}C AND ALDICARB SULFONE-^{14}C AT A RATE OF 20.0 PPM IN THE DIET

	12 hr				7 days		
Metabolites	Breast	Gizzard	Liver	Kidney	Breast	Gizzard	Liver
Aldicarb sulfoxide	0.0	0.0	3.0	0.0	0.0	0.0	0.0
Oxime sulfoxide	9.3	6.4	2.6	4.1	0	0	0
Oxime sulfone	19.7	23.1	14.4	22.0	0	0	0
Nitrile sulfoxide	8.1	5.7	10.1	8.2	0	0	0
Nitrile sulfone	412.6	410.4	418.0	401.6	144.1	74.2	184.5
Alcohol sulfoxide	2.5	2.4	0	0.7	0	0	0
Alcohol sulfone	3.0	3.9	10.6	10.6	0	0	0
Unknown 5	6.4	6.1	7.2	7.1	0	0	0
Hexane-solubles	5.3	13.3	95.4	96.5	4.0	12.0	19.8
Water-solubles	80.0	98.5	160.3	321.6	22.7	15.1	29.0
Unextractables	138.7	242.8	681.9	503.3	112.4	231.5	129.3

Aldicarb in Boll Weevils and Houseflies

The current study was conducted so that the metabolism of aldicarb in insects could be compared with that reported for mammals. Boll weevils, Anthonomus grandis Boheman, and houseflies, Musca domestica L., were used as test insects in order that species differences among insects also could be considered.

Methods

Insects used in this investigation were from insecticide-susceptible strains which had been maintained in the laboratory for several years. Only female houseflies were selected for testing, but both male and female weevils were included. All insects were utilized within 3 to 7 days after they had reached the adult stage.

For metabolism studies, aldicarb-carbonyl-C^{14} was applied topically to the insects in 1 microliter of acetone. Technical grade, non-radioactive aldicarb was used in toxicity tests and in cholinesterase, ChE, assay experiments. The ChE assay procedure of Simpson et al. (1964) was employed.

Following treatment of the flies or weevils with aldicarb-carbonyl-C^{14} at a dosage rate of 0.1 microgram per insect, groups of 20 insects were placed in 250 ml Erlenmeyer flasks until analyzed. Each flask was fitted with a side arm to allow a continuous flow of air into the chamber. Air was drawn through the flask and a carbon dioxide trap. This allowed the collection and quantitation of carbon-14 dioxide which was indicative of the rate of hydrolysis of the insecticide. At predetermined times after treatment, the radioactivity on the surface of the insects, that located in the body and that excreted was determined. Methods of extraction, chromatographic purification and identification of metabolites were the same as those described by Andrawes et al. (1967).

Results and Discussion

There was a rapid disappearance of aldicarb-carbonyl-C^{14} from the surface of boll weevils and houseflies following topical application of the insecticide (Table 17). By 6 hours after treatment, about 6% of the applied dose was detected in the external wash of the houseflies; thereafter, no radioactivity could be detected. With the boll weevils, approximately 30% of the dose remained on the surface 6 hours after treatment. Subsequent analysis showed that trace amounts of radioactivity could still be detected in the surface wash of weevils after 24 hours.

Some of the loss of insecticide from the surface probably resulted from mechanical removal as the insects came in contact with the holding containers. Thus, a portion of the aldicarb, per se, found in the containers may have come from this source and not actually have been excreted by the weevils or flies.

Data in Table 17 show that the amount of aldicarb-C^{14} equivalents within the insects remained relatively constant during the first 6 hours after treatment. This indicated that the rate of excretion was sufficient in both insect species to prevent large build up of the insecticide when applied at a sub-lethal dose.

Considering all the distribution data, it was evident that houseflies absorbed and excreted the carbamate at a faster rate than the boll weevils. The reason for the faster excretion rate in houseflies was evident by the nature of the radioactivity in the excreta. For example, 26% of the aldicarb-C^{14} equivalents in the excreta of flies were in the form of water soluble metabolites after 6 hours; in boll weevils, they constituted less than 4%. Although the water soluble metabolites are listed as unknown metabolites in Table 17, it is suspected that they are conjugates of some type (Andrawes et al. 1967). This type of metabolite represents a near end-point in the metabolism of carbamate materials and is the form most easily eliminated from the body. The greater ability of the flies to convert aldicarb to water soluble metabolites also was evident by their concentrations in the internal extracts of the insects.

Aldicarb sulfoxide was the major non-conjugated metabolite formed in boll weevils and houseflies. Further oxidation yielded lesser amounts of the sulfone derivative. Hydrolytic products were not detected in any of the extracts in either of the insect species. This was not unexpected since the amount of carbon-14 dioxide produced was very minute. The percentages of the applied doses liberated as carbon-14 dioxide were 0.5 and 1.0 after 6 hours in the weevils and flies, respectively.

The inability of the boll weevils and houseflies to rapidly hydrolyze aldicarb and its carbamate metabolites was the major difference observed in the metabolism of aldicarb by insects and rats. Hydrolysis was the predominant pathway of metabolism in rats treated orally with the carbamate. In fact, over 50% of the dose was hydrolyzed within 8 hours after treatment (Andrawes et al. 1967). Consequently, most of the metabolites formed in rats were non-toxic derivatives of aldicarb such as oxime sulfoxide and nitrile sulfone.

Since aldicarb sulfoxide was formed very rapidly and its concentration remained relatively constant in the bodies of the insects over a 6-hour period, attempts were made to estimate its contribution to the insecticidal activity of aldicarb. First, the LD_{50} of aldicarb and aldicarb sulfoxide to boll weevils and houseflies when applied topically was determined. With the boll weevil, the LD_{50} value was 10 micrograms per gram for aldicarb and 150 micrograms per gram for aldicarb sulfoxide. LD_{50} values for the 2 compounds with houseflies were 5 and 12.5 micrograms per gram, respectively. These data demonstrated that aldicarb sulfoxide could contribute to the insect toxicity of the parent compound.

Studies of the anticholinesterase activity of aldicarb and aldicarb sulfoxide against housefly-head ChE and boll weevil whole-body ChE indicated that the toxicity of aldicarb sulfoxide might be more important than indicated by the LD_{50} tests. Whereas the I_{50} values of aldicarb against fly ChE was $4.7 \times 10^{-6}M$, the value was $3.0 \times 10^{-7}M$ for aldicarb sulfoxide. The values obtained for boll weevil ChE were about the same. It is evident, then, that the aldicarb sulfoxide is a much more potent ChE inhibitor than the parent compound and that it should be more active as an insecticide. That this was not demonstrated by topical applications to the insects may be that the sulfoxide degrades on the surface of the insects faster than does aldicarb, or that the penetration rate is much slower than that of aldicarb. In either case, the compound could not exhibit its full toxic potential when applied topically. However, it may be more insecticidal than aldicarb when formed in the body as a result of enzymic metabolism.

TABLE 17. DISTRIBUTION AND EXCRETION OF ALDICARB AND ITS METABOLITES IN BOLL WEEVILS (W) AND HOUSEFLIES (F) TREATED TOPICALLY WITH ALDICARB-CARBONYL-C^{14}

	Percent of recovered radioactivity[a]/hours							
	1		2		4		6	
Metabolites	W	F	W	F	W	F	W	F
				Surface				
Aldicarb	57.6	13.6	39.9	13.7	25.5	11.8	19.6	4.2
Aldicarb sulfoxide	5.4	5.4	5.5	5.9	7.8	6.5	9.8	2.4
				Internal				
Aldicarb	11.2	18.7	11.1	15.2	12.5	14.0	13.5	8.9
Aldicarb sulfoxide	2.8	16.1	6.3	16.5	5.6	13.1	6.6	7.2
Aldicarb sulfone	1.0	6.1	2.8	6.4	4.1	7.8	5.4	4.5
Unknown[b]	1.2	15.8	2.8	16.2	5.6	17.7	5.9	14.6
				Excreted				
Aldicarb	12.3	12.2	12.3	10.4	13.7	10.4	13.4	10.0
Aldicarb sulfoxide	6.5	5.1	13.5	5.5	15.0	5.6	14.3	13.3
Aldicarb sulfone	0.6	0.7	3.0	1.8	6.8	1.8	7.6	9.3
Unknown[b]	0.7	6.3	2.8	9.4	3.4	11.3	3.9	25.6

[a] The recovery of the applied dose ranged from 83 to 98%, with an average of 92%.

[b] Radioactive metabolites in the water phase after partitioning between water and chloroform.

Carbaryl in Lactating Cows

The evaluation of the residual nature of carbaryl in cattle has been a major source of progress in defining the metabolic fate of carbamate insecticides. Even before the realization that oxidative and hydroxylative mechanisms played a significant role in the metabolism of carbaryl, some of the more complete fate studies with this carbamate were performed with cows (Gyrisco et al., 1960; Claborn et al., 1967; Whitehurst et al., 1963). Although the analytical methods used by these scientists were later reported to be inadequate for the detection of total carbaryl residues in meat and milk (Dorough, 1967) they did establish that carbaryl was rapidly metabolized and excreted by dairy animals.

In 1964, a report on the metabolism of carbaryl indicated that the biochemistry of this carbamate, and probably carbamates in general, was a very complex subject (Dorough and Casida, 1964). These authors demonstrated the presence of certain carbaryl metabolites in the milk of a treated goat that were neither carbaryl or 1-naphthol nor any product which could be converted into 1-naphthol by alkaline hydrolysis. Subsequent studies on the metabolism of carbaryl in dairy cows were instrumental in confirming the identity of some carbaryl metabolites and in suggesting the chemical nature of others isolated for the first time (Baron et al.,1968, 1969; Dorough, 1967). Progress towards the complete elucidation of the metabolic fate of carbaryl in cows and other animals has recently been reviewed (Dorough, 1970).

General Study Plan

A summary of the overall study is presented in Table 18. One Holstein cow was used at each of the feeding levels indicated. All animals were in a medium stage of lactation, and production was considered satisfactory for a commercial dairy. They were housed in metabolism stalls, which permitted separate and quantitative collection of urine and feces. Non-radioactive carbaryl was administered to the animals for a two-week period before feeding of the ^{14}C-labelled carbamate began. Collection of samples for analysis began 12 hr after the first carbaryl-^{14}C treatment.

The 5 p.p.b. level of sensitivity for carbaryl-^{14}C equivalents was determined on the basis of the specific radioactivity of the parent compound, which was 1.2 mc/mmole, and the size of sample radioassayed. For milk, 0.5 g was routinely assayed, and 1 g dry weight of tissue was assayed. The sensitivity was increased when necessary by assaying larger samples. The basic procedures used for quantitation of residues and for their isolation and identification were similar to those described in a study involving aldicarb (Dorough et al., 1970).

Elimination of Carbaryl

As expected from the earlier studies on the metabolism of carbaryl in cows, there was very rapid elimination of the doses from the animals. It was evident that an equilibrium between 'intake' and 'output' of carbaryl was established in rather fast order. Generally, the percentage of the previously applied dose present in the excreta remained fairly consistent after three or four days of feeding the radioactive compound.

Milk from cows fed 10, 30 or 100 ppm carbaryl contained about 0.2 per cent of the consumed carbamate. There were no indications that the pattern of excretion was altered significantly by increasing the dose tenfold. It was noted that the residues in the milk of the cow fed 100 ppm did not reach their maximum levels as quickly as they did in animals fed the two lower doses. However, this may have been caused by some biochemical difference in this particular animal since carbaryl-^{14}C equivalents in the urine and feces exhibited a similar excretion pattern.

Most of the administered carbaryl doses were eliminated from the body in the urine. From 70 to 85 per cent of the consumed carbamate was detected in the urine. There was a correlation between dose and percentage of dose excreted in the urine, with more complete elimination occurring as the dose decreased. The opposite pattern was indicated in the feces, where 5 to 11 percent of the dose was excreted. This type of excretion suggested that the metabolism of carbaryl may have been hindered somewhat by the larger levels of toxicant consumed. However, it is important to note that the total elimination of carbaryl equivalents exceeded 80 percent of the administered doses regardless of the level fed to the animals.

Carbaryl-^{14}C equivalents in the milk as ppm - Having established that the percentage of the carbaryl doses eliminated in the milk remained at a fairly constant level during the 14-day study, it was expected that the same would hold true when the residues were converted into ppm in the milk. Generally this was found to be the case. However, there was some day-to-day variation in milk production, which resulted in slight variations in the ppm level observed in the milk. These minor variations were expected but were not considered too important since there was no indication that carbaryl-^{14}C equivalents were increased as the time of feeding was extended.

Therefore our investigations showed that the level of total carbaryl-^{14}C equivalents in the milk of cows on a continuous diet containing carbaryl was approximately 1/400 of that level in the diet. The non-accumulative nature of this carbamate in milk was likewise observed when aldicarb was fed to cows for 14 days (Dorough et al., 1970).

Extraction of carbaryl metabolites from milk - From a practical and toxicological viewpoint, the numbers defining the total carbaryl equivalents in milk have little significance. What is important, however, is the amount of those equivalents that were toxic or which should be considered toxic until proven otherwise. Generally, the latter would include any metabolite having the carbamate moiety still attached to the naphthyl ring and any other metabolites of unknown identity.

In the previous studies on the metabolism of radioactive carbaryl in dairy cows, the milk was extracted with organic solvent and the total residues in the organic extract, water phase and milk solids were quantitated. Only those metabolites appearing in the organic solvent phase have been subjected to rigorous testing to establish their identity. Such techniques were effective in identifying, or tentatively identifying, less than one-half of the total residues (Dorough, 1967).

A major portion of our efforts in the current study was devoted to the development of an extraction procedure that would remove all residues from the water and solid phases of milk. Finally, a procedure that accomplished this goal was perfected.

Acetone was added to 50 ml of milk contained in a glass-stoppered, 250 ml Erlenmeyer flask and the contents were thoroughly shaken before addition of the acetonitrile. Reversing the order, or adding the two solvents together, coagulated the milk proteins so rapidly that residues were trapped in the solids, and additional extractions with either acetone or acetonitrile were ineffective in their removal. The acetonitrile, after the acetone, coagulated the milk proteins without trapping the carbaryl residues and allowed the proteins to be removed by filtration. However, the flask and solids were thoroughly washed with acetonitrile. Analysis of 1 g of the dried milk solids by combustion techniques revealed that they were free of radioactive residues.

The filtrate was added to a separatory funnel and extracted with hexane to remove the fats and oils. After back-extracting the combined hexane with 20 ml of acetonitrile, the hexane was discarded. The acetonitrile was returned to the separatory funnel containing the milk extract.

Once all carbaryl-^{14}C equivalents were removed from the milk solids, the most important step in this procedure was the removal of the water while retaining the residues in the organic solvent phase. This was done by adding sodium chloride to the extraction mixture, slowly with shaking, until two layers were formed. Chloroform (50 ml) was then added and the funnel shaken vigorously, and then the layers again were allowed to separate completely. Chloroform was necessary in this procedure because considerable water was present in the acetonitrile-acetone layer when chloroform was not used. Without the addition of the salt, the more polar metabolites were only partially extracted.

The water layer was extracted twice more with acetonitrile and chloroform and the water phase then discarded. No radioactive residue was detected in a 1 ml aliquot of the water, and only traces were evident when the entire water layer was concentrated and radioassayed directly. Thus all the carbaryl equivalents had been extracted from the milk and were present in an organic solvent.

The extract of the milk was then dried with anhydrous sodium sulphate and concentrated just to dryness on a rotary evaporator. Small washes of acetonitrile were used to transfer the residue to a 15 ml centrifuge tube. If the extract appeared to contain too much oily material for application to a thin-layer chromatogram, the acetonitrile was extracted with 2-4 ml of hexane. This step was usually necessitated by impure solvents rather than by oils from the milk.

Recent work has shown that the butterfat of milk is free of carbaryl metabolites. Therefore skim milk can be used without any changes in results and with less oil to be removed by the hexane washes.

Separation and isolation of residues in milk - A series of thin-layer chromatographic (tlc) analyses, on Chromar 500 sheets, was used to separate and help identify the radioactive residues from the milk. The entire extract was applied to a chromatogram and developed two-dimensionally. Radioautograms of the tlc revealed that six radioactive components were resolved by the solvent systems used. Each of the areas above the origin was isolated separately and analysed in several two-dimensional solvent systems. These analyses demonstrated that each radioactive area, II-VI, was composed of a single material. Metabolites II-VI are those referred to in previous studies as the 'organo-solubles'. The radioactive materials remaining at the origin of tlc no. 1 were a combination of the 'water-solubles' and 'solids or unextractables' that were obtained with extraction procedures reported earlier (Dorough, 1967).

The tlc no. 1 origin material was extracted with methanol and then applied to a new tlc and developed in ethyl acetate. When this was done, the radioactive material was resolved into two distinct areas (tlc no. 2). Since such polar metabolites of carbaryl and other carbamates have been shown to be conjugates of some type (Dorough, 1970), metabolites VII and VIII were extracted from the tlc no. 2 and subjected to Glusulase enzyme. This enzyme contained both β-glucuronidase and sulphatase and therefore would cleave the metabolites if they were glucuronide or sulphate conjugates.

To determine if cleavage would occur, an aliquot of the methanol extract of either metabolite VII or VIII was added to a 25 ml Erlenmeyer flask and the solvent removed by evaporation. Citrate-phosphate buffer pH 5.0 (4 ml), and 0.5 ml of the Glusulase solution, as received (Endo Lab. Inc., Garden City, New York 11530), was added to the flask. A drop of toluene was added to retard microbial growth and the flasks were incubated, with shaking, at 37°C for 24 hr.

After incubation the contents of the flask were transferred to a separatory funnel with water washes of the flask until the final volume was approximately 20 ml. Extraction of the radioactive products from the water was accomplished by using the procedure described for the extraction of milk.

The concentrated extract was applied to a tlc and developed in methylene chloride-ethyl acetate (1:1). Radioautographic analysis of the tlc's showed that metabolite VII yielded two aglycones, VII A and VII B, and that metabolite VIII yielded only one product, VIII A. Cleavage of metabolites VII and VIII by Glusulase enzyme was about 60 to 70 percent during the 24 hr period of incubation. Repeated isolation of the non-cleaved material and additional incubation with Glusulase enzyme showed that metabolites VII and VIII could be converted almost quantitatively into the aglycones.

Over 90 percent of the applied radioactivity was routinely recovered from tlc no. 1 and no. 2. Therefore the metabolites on these tlcs were not unusually volatile, and no special precautions were required for good recovery and re-chromatography. However, metabolite III was often converted almost completely into metabolite IV. This conversion was kept to a minimum by using very pure solvents and maintaining metabolite III under slightly acid conditions whenever possible.

Poor recoveries of metabolites VII A and VIII A from tlc no. 3 were often encountered when attempts were made to isolate them for futher study. This was primarily a function of volatility during the evaporation of solvents since direct counting of the radioactive areas of the tlc yielded recoveries exceeding 80 to 90 percent of the applied materials.

Chemical nature of residues in milk - Once techniques for separating and isolating carbaryl metabolites from milk had been accomplished, each material was subjected to a variety of tests to determine its identity. The small concentrations of certain of the metabolites precluded detailed analysis of their chemical nature. When the same metabolite also was present in cow urine, but at much higher levels, that material was isolated and used for identification purposes. This was the case for metabolite II (and its hydrolytic product not detected in milk) and metabolites III and IV.

Results of the characterization studies of the metabolites in milk are shown in Table 19. A summary of the data supporting these findings is presented below.

Metabolite III- This product is considered first because it was the major metabolite of carbaryl in the milk and because its chemical characteristics were important in the identification of metabolite II. Moreover, it has been tentatively identified in a number of organisms in the past, and its identity after isolation from milk has been recently confirmed (Baron et al., 1968, 1969).

The basis on which our particular metabolite III from milk was identified as 5,6-dihydro-5,6-dihydroxy-1-naphthyl methylcarbamate were: (a) metabolite II cochromatographed with authentic sample synthesized by Union Carbide chemists; (b) both the material from milk and the synthetic product were identical and supported the proposed structure.

Metabolite II - This metabolite and its hydrolytic product were found in rather large quantities in the urine. Therefore, these materials were isolated from the urine in sufficient quantity for mass spectral analysis. Since 3,4-dihydro-3,4-dihydroxy-1-naphthyl methylcarbamate has not been synthesized, identification of metabolite II as this product was based largely on its similarity to the 5,6-dihydrodihydroxy derivative of carbaryl. In addition to the fact that the mass spectral data were consistent with the proposed structure, metabolite II could be dehyrated to yield 4-hydroxy-1-naphthyl methylcarbamate. This reaction was comparable to that already mentioned in reference to metabolite III.

Metabolites V and VI - Each of these two materials was present at low levels in the milk, and the evidence for their structures was based primarily on cochromatography with authentic samples. There was sufficient quantity of metabolite VI to establish that it yielded 1-naphthol upon hydrolysis, which further supported its identity as carbaryl.

Metabolite VIII - This metabolite was identified as 1-naphthyl sulphate. Before its incubation with Glusulase it cochromatographed with a standard sample of 1-naphthyl sulphate (potassium salt). That this product exists interchangeably as a number of salts was point out by Paulson et al., 1970. These salts chromatograph differently on tlc and can lead to confusion about the identity of the material. This can be avoided by converting the compound into the sulphate by the addition of acid, or by converting it into a single salt by adding any one salt in excessive amounts. Glusulase enzyme cleavage of metabolite VIII yielded the aglycone, 1-naphthol (metabolite VIII A on tlc no. 3), in the same manner as the standard sample.

Metabolite VII - This material represents an entirely different type of carbaryl metabolite and one which has not been previously isolated. It is a carbamate and is formed by two types of conjugation, namely, sulphate conjugation and methylation. Data to support its identity as 1-methoxy-5-(methylcarbamoyloxy)-2-naphthyl sulphate are as follows: (a) It is a conjugate metabolite. This is based on the polar nature of the metabolite and the fact that Glusulase enzyme treatment yields two aglycones, metabolites VII A and VII B. About 99 percent of metabolite VII is a conjugate of VII A. (b) It is a sulphate conjugate. Sulphatase hydrolysed twice as much as metabolite VII as did β-glucuronidase. By inhibiting the sulphatase activity in the glucuronidase preparation, the β-glucuronidase did not hydrolyse any of the conjugate metabolite. (c) Aglycone VII A is a ring-modified carbamate metabolite of carbaryl. If aglycone VII A was not a ring-modified derivative of carbaryl, alkaline hydrolysis would yield 1-naphthol. However, hydrolysis of VII A yielded a product that cochromatographed with VII B. Mass spectral analysis of aglycone VII A showed a loss of methyl isocyanate, which also demonstrated that the ring was modified and, more important, showed that the metabolite was definitely a carbamate material. Other peaks of the mass spectrum suggested the presence of an hydroxyl group and a methoxy group. Mass spectral and i.r. data on 5-methoxy-6-hydroxy-1-naphthol methylcarbamate synthesized by Union Carbide chemists were identical with that of aglycone VII A.

Aglycone VII B is probably the hydrolytic product of VII A. However, the yield of VII B from the enzyme hydrolysis of metabolite VII is so small that detailed studies have not been possible. It is known that alkaline hydrolysis of VII A does yield a product that cochromatographs with aglycone VII B. These data suggest the possibility that VII B is not present in milk as a conjugate but may form from aglycone VII A during the enzymatic cleavage of metabolite VII.

Residues in Meat - The total carbaryl-^{14}C equivalents in various tissues of cows fed with carbaryl for 14 days are shown in Table 20. The animals were slaughtered and the tissues taken approximately 18 hr after the last treatment with the insecticide. Total residues in each sample were determined by combusting 1 g of dry tissue and collecting the radioactivity as ^{14}C-labelled carbon dioxide.

All of the tissues from cows fed with carbaryl at 30 and 100 ppm contained detectable levels of residues. At the 10 ppm feeding level, all of the tissues contained residues except the fat. A good correlation existed between the level of pesticide fed and that which appeared in the tissues. Total carbaryl-^{14}C equivalents in the muscle were 1/1000 of that level fed in the diet.

Although the total radioactive content of the tissues was quite low, attempts were made to determine the nature of the residues in all tissues except the fat. Tissue (25 g) was homogenized with 50 ml of water and then 100 ml of acetonitrile was introduced and blending continued. Again, the blender was stopped, 25 ml of acetone added and homogenization continued for about 1 min. The acetone aided protein coagulation and facilitated filtering. After being filtered off on Whatman No. 1 paper, the solids were extracted once more. The extracts were combined and analysis was continued by using a procedure similar to that described for milk. Unlike those in milk, however, some radioactive residues remained in the tissue solids after extraction and in the water phase after the addition of sodium chloride and partitioning with acetonitrile and chloroform. The concentration of these unknown materials and those residues that were identified are shown in Table 21.

Carbaryl was detected in all tissues except the blood. It accounted for 17 percent of the carbaryl-^{14}C equivalents in the muscle but constituted a lesser proportion of the total residues present in other tissues.

The only other identified carbamate metabolite from the tissues was 5,6-dihydro-5,6-dihydroxy-1-naphthyl methylcarbamate. It was a major constituent of the residues in the muscle, heart and blood. Small quantities of the hydrolytic product of this metabolite were found in other tissues except the muscle and lungs.

Naphthyl sulphate was identified as 52 percent of the carbaryl-^{14}C equivalents in the blood. The same material accounted for about 30 percent of the residues in the kidney and lung but was low in the liver and heart; none of this product was evident in the muscle tissue.

Extraction of the liver and kidney with various solvents and for different periods of time failed to remove more of the radiocarbon than did the acetonitrile-water-chloroform system. Homogenization in water alone appeared to extract more of the ^{14}C-residues from the tissues, however, the addition of any organic solvent caused a precipitate to form which contained large quantities of radioactivity.

Ultrasonic disintegration of the tissues (Brinkmann Polytron) in ethanol, followed by overnight soxhlet extraction with the same solvent removed 20% from the kidney. Soxhlet extraction for another 24 hrs using acidified ethanol was ineffective.

Separation of liver and kidney homogenates into various centrifugal fractions was conducted and the distribution of radioactivity among the cellular components evaluated. Homogenization of the tissue, 25 g in 200 ml of water, was accomplished using a Virtis homogenizer. The homogenate was centrifuged at 15,000g for 30 min., the supernatant decanted and the pellet resuspended in 100 ml of water. After centrifugation at 15,000g for 30 min and the supernatant decanted, the pellet was again suspended in water and centrifuged. The supernatant fractions from each centrifugation was radioassayed separately by liquid scintillation counting. The first supernatant was subjected to additional centrifugation at 105,000g for 1 hr and the supernatant decanted and radioassayed. Both the 15,000g pellet and the 150,000g pellet were radioassayed by combusting the solids in a Beckman Biological Materials Oxidizer.

Distribution of the ^{14}C-residues among the various cellular fractions of liver and kidney are shown in Table 22. In each case, from 80 to

85% of the radioactivity was in the 15,000g soluble fraction. Centrifugation at 105,000g did not remove any of the radiocarbon from the 15,000g solubles of the liver, but 1.4% of the radioactivity in the kidney was detected in the microsomal (105,000g pellet) fraction.

Numerous modifications of the procedures used in the centrifugal fractionation of the liver and kidney were attempted. However, the results were always similar to those presented in Table 22. For example, centrifungation of the tissue homogenates at 5,000g or 15,000g showed that the resulting pellet contained essentially the same levels of radioactivity. Re-homogenation of the pellets, as opposed to washing by suspending the pellets in water, did not increase the level of ^{14}C-residues in the supernatant fractions. Also, phosphate buffer, pH 7.0, was shown to work as well as water, but no better. The use of the buffer would be desired when the supernatants are to be exposed to enzymatic treatment.

Repeated ultrasonic homogenization of the tissues in water or buffer did not alter the distribution pattern of the ^{14}C in the liver and kidney. However, the procedure is faster than homogenization in the Virtis. Three successive 40-sec. homogenizations (10g in 80 ml) of the tissue yielded results almost identical to those presented in Table 22.

Organic solvent extraction of the supernatant and pellet - Combined supernatants and pellets of the liver and kidney were extracted using the acetonitrile-chloroform procedure previously described.

Following extraction of the liver supernatant, 40.1% of the ^{14}C in the whole tissue was detected in the precipitate which formed upon addition of the organic solvents. The water phase contained 19.8% and the acetonitrile-chlorofrom phase contained 21.4%. In the kidney, 12.8% of the ^{14}C in the whole tissue was in the precipitate or solids, 34.8% in the water and 30.8% in the organic solvent phase. The distribution of radioactivity among the 3 phases was changed only slightly by refluxing the supernatants for 3 hrs. after adjusting to 3N HCl (Table 23). Recent studies have indicated that a weaker acid will convert more of the radiocarbon to the chloroform-acetonitrile layer.

The extraction characteristics of the ^{14}C-residues associated with the 15,000g pellets of the liver and kidney are shown on Table 24. These data were obtained from tests where the pellets were suspended in 3N HCl and refluxed for 3 hrs. However, distribution of the radioactivity among the 3 phases was essentially the same when the acid treatment was omitted.

Combined results of the extraction of the supernatant (Table 23) with those of the extraction of the 15,000g pellets (Table 24) are as follows:

	% of total ^{14}C in sample		
	Solids	Water	Organo-extractables
Liver	45	27	27
Kidney	11	46	44

When compared with the data obtained upon direct extraction of the tissues, it may be seen that the final extraction characteristics were unchanged by separating the ^{14}C-residues according to cellular fraction. However, these latter studies do show that a maximum of 9% of the radiocarbon in the liver and 5% of that in the kidney cannot be removed from components of the cell found in the 15,000g pellet. The remainder of the ^{14}C-residues was extracted from the tissues and may be generally classified as organo-extractables and water solubles. The total organo-solubles after acid treatment accounted for approximately 30% of the radiocarbon in the liver and 45% of that in the kidney. Water soluble ^{14}C-residues constituted 63% of the radiocarbon in the liver; about 47% of these (30% of total ^{14}C in liver) was as materials precipitated from the 15,000g supernatant with solvent, 43% (27% of total ^{14}C) as materials remaining in solution after addition of solvent, and 10% (3% of total ^{14}C) as materials removed from the 15,000g pellet by extraction with acetonitrile and water. Water soluble ^{14}C-residues constituted 52% of the radiocarbon in the kidney; about 11% (6% of total ^{14}C) was as materials precipitated from the 15,000g supernatant with solvent, 74% (40% of total) as materials remaining in solution after addition of solvent to the 15,000g supernatant, and 15% (7% of total) as materials removed from the 15,000g pellet by extraction with acetonitrile and water. Based on these data and the report by Dorough (1971), a preliminary hypothesis relative to the nature of the ^{14}C-metabolites in the liver and kidney might be suggested as follows:

Nature of ^{14}C-metabolite	% of total ^{14}C in tissue	
	Liver	Kidney
Free: Carbaryl, dihydrodihydroxy-carbaryl, etc.	16	10
Conjugated:		
O-Glucuronides, O-Sulfates, etc.	12	33
Glutathione conjugates	27	46
Protein bound and/or incorporated	36	6
Bound to cellwall and/or membranes	9	5

Protein precipitation and dialysis- In order to prepare the 15,000g supernatant for more detailed analysis such as tlc, electrophoresis, etc., it will be necessary to remove most of the protein from the solution. This was attempted with the liver 15,000g supernatant using several methods which would precipitate the proteins (Table 25). All of the methods which precipitated the majority of the proteins (heat, acetonitrile, ammonium sulfate) resulted in 40% or more of the radiocarbon in the precipitate. Acetone, up to 30%, precipitated most of the protein without loss of any of the radioactivity from the supernatant. However, precipitation with loss of radiocarbon to the solids occurred during the process of removing the acetone by evaporation. Because of the limited amount of liver available, no further studies of this nature have been performed with this tissue. It is of interest, however, that heat, acetonitrile and 40% ammonium sulfate resulted in 40 to 48 percent of the radiocarbon in the supernatant being precipitated with the proteins. This is equivalent to approximately 35% of the total ^{14}C-content of the liver and agrees very well with the level, 36%, hypothesized to be bound and/or incorporated with protein.

Further studies of protein precipitation using the kidney 15,000g supernatant showed that 65% of the radiocarbon which was precipitated with ammonium sulfate could be dialyzed (Table 25). Therefore, it is unlikely that ammonium sulfate or the other methods of precipitation selectively precipitated only that radiocarbon bound to or incorporated into various proteins.

Tlc analysis of the supernatant after ammonium sulfate precipitation showed 4 distinct bands (EM precoated silica gel plates, F-254, 0.25 mm, developed in 75:15:10 chloroform-methanol-acetic acid). Fifty-six percent of the radioactivity applied to the plate remained at the origin, 28% chromatographed identical to naphthyl glucuronide, 9% chromatographed identical to naphthyl sulfate and 7% moved to the solvent front as did standards of carbaryl, 1-naphthol and other free metabolites. The fact that certain of the radioactivity chromatographed the same as naphthyl glucuronide and sulfate suggests that the ^{14}C-materials were of this nature. However, it is not suggested that the compounds were only naphthyl glucuronide or only naphthyl sulfate. While this tlc system has been shown to effect separation of sulfate and glucuronides, it has not been effective in separating mxitures of sulfates or mixtures of glucuronides.

The dializate from the 75-95% ammonium sulfate precipitation (Table 26, 76% dialyzed) was also analyzed by tlc as described above. In this case, 27% of the radioactivity remained at the origin, 16% chromatographed identical to naphthyl glucuronide, 45% identical to naphthyl sulfate, 5% as a band just above the sulfate, and 7% moved to the solvent front. These precipitation and dialysis data demonstrate the care which must be taken in future studies if complete separation of the metabolites is to be achieved.

Sephadex column chromatography - A Sephadex G-100/120 column was prepared by suspending 20 g of the gel in 400 ml of distilled water and allowing to swell for 72 hours. A 3 cm, id, column was packed to a height of 46 cm with the Sephadex and equilibrated with phosphate buffer, pH 7.0.

The 15,000g supernatants of liver or kidney were added to the column (homogenate of 20-25 g of tissue concentrated to 10 ml) and eluted with 500 ml of phosphate buffer. Ten to 15 ml fractions were collected and 0.5 ml aliquots from each fraction radioassayed.

With both the liver and kidney, only 2 radioactive peaks were eluted from the column, with recovery of the added radiocarbon being greater than 95%. The first radioactive peak (I) began to elute from the column in the 8th fraction and continued through the 23rd fraction, with the maximum concentration occuring in the 17th fraction. Peak II was eluted from the column in fractions 26 through 36, with fraction 31 containing the greatest concentrations of radiocarbon.

Of the total radiocarbon recovered from the column, peak I contained 49% in the case of the liver and only 13% in the case of the kidney. The remainder, 51 and 87% respectively for the liver and kidney, was contained in peak II. The nature of these materials have yet to be critically evaluated. However, it could be that the peak I materials are the same as the "Unextractable Unknowns" reported by Dorough (1971) and the peak II was a combination of the "free metabolite" and "water-soluble unknowns". This is based on the fact that the distributions of the ^{14}C-residues in the liver and kidney were very similar when examined by the 2 different methods. For example, the "Unextractable Unknowns" represented 48 and 18% of the radiocarbon in the liver and kidney when extracted with acetonitrile-chloroform, whereas peak I represented 49 and 13% of the radiocarbon in the 15,000g supernatant of the 2 tissues.

When a portion of peak II from the kidney was extracted directly with acetonitrile-chloroform, 22% of the radioactivity was recovered in the organic solvent phase. Tlc analysis (EM F-254 plates developed in 4:1 methylene chloride-acetonitrile) showed that approximately 15% of the radioactivity remained at the origin and 85% chromatographed identical to a 5,6-dihydrodihydroxycarbaryl standard. No cochromatography of the 2 materials was attempted. Acid treatment of another portion of peak II (1N HCl, 37°C for 16 hrs) prior to extraction resulted in equal portions of radioactivity in the water and organic solvent phases. Tlc of the organo-solubles showed 3 radioactive bands, 1 in the area of 5,6-dihydrodihydroxycarbaryl (15%), 1 in the area of 5,6-dihydrodihydroxynaphthol (15%) and 1 chromatographing in the area of carbaryl. The use of standards to indicate location of the radioactive bands is not intended as tentative identification but only to point out the general behavior of these materials on tlc. Many analogs of carbaryl chromatograph similar to the standards mentioned here. This is especially true for the area where carbaryl, per se, is located. Detailed examination of the tlc characteristics of the materials in a number of solvent systems must be accomplished before even tentative identification can be made.

TABLE 18. STUDY PLAN FOR CARBARYL-NAPHTHYL-^{14}C COW FEEDING EXPERIMENT.

Dietary dose (ppm)	0.10,30,100
Treatment method	Gelatin capsule (one every 12 hr)
Treatment schedule	14 days on 'cold' carbaryl followed by 14 days on carbaryl-naphthyl-^{14}C
Sampling	Milk, urine, feces: every 12 hr, Tissues: 18 hr after last dose
Sensitivity	0.005 ppm carbaryl-^{14}C equivalents

TABLE 19. CHEMICAL NATURE OF CARBARYL METABOLITES IN COW'S MILK AND THEIR AVERAGE CONCENTRATIONS AFTER FEEDING WITH CARBARYL (100 PPM IN THE DIET FOR 14 DAYS)

Metabolite No.	Chemical nature	Amount in milk (ppb)	% of total
VI	Carbaryl	17	6
II	3,4-Dihydrodihydroxy-1-naphthyl methylcarbamate	13	6
III	5,6-Dihydrodihydroxy-1-naphthyl methylcarbamate	94	34
V	5-Hydroxy-1-naphthyl methylcarbamate	3	1
IV	5,6-Dihydrodihydroxynaphthalene	9	3
VIII	1-Naphthyl sulphate	72	26
VII A	1-Methoxy-5-(methylcarbamoyloxy)-2-naphthyl sulphate	63	23
VII B	5-Methoxy-1,6-naphthalenediol	7	2

TABLE 20. TOTAL CARBARYL-^{14}C EQUIVALENTS IN COW TISSUES AFTER FEEDING WITH CARBARYL-NAPHTHYL-^{14}C (10, 30 AND 100 PPM IN THE DIET FOR 14 DAYS)

	Carbaryl-^{14}C equivalents (ppb)		
Tissues	10 ppm	30 ppm	100 ppm
Kidney	0.095	0.531	1.003
Liver	0.033	0.100	0.411
Lung	0.020	0.064	0.027
Muscle	0.009	0.031	0.104
Heart	0.012	0.038	0.095
Fat	0.000	0.015	0.025
Blood	0.008	0.036	0.141

TABLE 21. RADIOACTIVE RESIDUES IN COW TISSUES AFTER FEEDING WITH CARBARYL-^{14}C (100 PPM IN THE DIET 14 DAYS)

Metabolites	% of total radioactivity in sample					
	Kidney	Liver	Lung	Muscle	Heart	Blood
Carbaryl (No. VI)	3.3	9.2	2.1	17.0	3.7	0
5,6-Dihydrodihydroxy carbaryl (No. III)	4.5	3.0	8.8	38.6	31.3	22.0
5,6-Dihydrodihydroxy naphthol	1.8	4.1	0	0	4.9	2.0
Naphthyl sulphate (No. VIII)	29.3	4.1	27.3	0	4.0	51.8
Water-soluble unknowns	43.2	32.9	47.5	30.6	41.8	7.1
Unextractable unknowns	17.9	46.7	14.3	13.8	14.3	17.1

TABLE 22. CENTRIFUGAL FRACTIONATION OF ^{14}C-RESIDUES IN THE LIVER AND KIDNEY OF A LACTATING COW FED 100 PPM CARBARYL-1-NAPHTHYL-^{14}C IN THE DIET FOR 14 DAYS

Fraction	Distribution, % of total ^{14}C in tissue[a]	
	Liver	Kidney
15,000g supernatant, I	75.1	66.7
15,000g supernatant, II	6.8	12.3
15,000g supernatant, III	2.6	3.8
Total 15,000g supernatant	85.1	82.8
105,000g supernatant	85.1	81.4
105,000g pellet	0	1.4
15,000 pellet	14.7	19.3
Recovery	99.8	102.1

[a] Comparable results were obtained when tissues were homogenized in distilled water or in phosphate buffer, pH 7.0.

TABLE 23. EXTRACTION CHARACTERISTICS OF ^{14}C-RESIDUES IN ACID-TREATED 15,000g SUPERNATANT OF LIVER AND KIDNEY HOMOGENATES (SEE TABLE 22)[a]

Fraction	Distribution, % of total ^{14}C in supernatant[b]	
	Liver	Kidney
Chloroform-acetonitrile	30.5 (26.0)	45.9 (37.4)
Water	27.2 (23.1)	47.9 (39.0)
Solids	42.0 (35.7)	7.4 (6.0)
Recovery	99.7	101.2

[a] Supernatant adjusted to 3N HCl and refluxed for 3 hrs.

[b] Numbers in parenthesis represent % of ^{14}C in tissue.

TABLE 24. EXTRACTION CHARACTERISTICS OF ^{14}C-RESIDUES IN ACID-TREATED 15,000g PELLET OF LIVER AND KIDNEY HOMOGENATES (SEE TABLE 22)[a]

	Distribution, % of total ^{14}C in pellet[b]	
Fraction	Liver	Kidney
Chloroform-acetonitrile	8.1 (1.2)	38.1 (7.4)
Water	28.6 (4.2)	36.7 (7.1)
Solids	60.3 (8.9)	23.3 (4.5)
Recovery	97.0	98.1

[a] Pellet placed in 3N HCl and refluxed for 3 hrs.

[b] Numbers in parenthesis represent % of ^{14}C in tissue.

TABLE 25. ^{14}C-RESIDUES IN THE PRECIPITATE OF A 15,000g SUPERNATANT OF LIVER HOMOGENATE TREATED IN VARIOUS WAYS TO EFFECT PRECIPITATION OF THE PROTEINS

Conditions for precipitation	^{14}C in precipitate, % of total ^{14}C in supernatant
Sodium chloride, saturated	25
Heat, 90°C for 0.5 hr	48
Acetonitrile, 20%	39
Acetonitrile, 40%	39
Acetone, 20%	0
Acetone, 30%	0
Acetone, 35%	12
Ammonium sulfate, 40% of saturation	46
Ammonium sulfate, 80% of saturation	68
Ammonium sulfate, 100% of saturation	73

TABLE 26. AMMONIUM SULFATE PRECIPITATION, AND DIALYSIS OF THE PRECIPITATE, OF A 15,000g SUPERNATANT OF KIDNEY HOMOGENATE

$(NH_4)_2SO_4$ % of saturation	% ^{14}C precipitated	mg protein ppt	Specific activity of protein, dpm/mg	% dialyzed	Specific activity of protein after dialysis
0-40	10.5	382.9	29	68.1	7.8
40-60	13.2	397.8	35	61.3	13.1
60-75	12.7	359.7	38	58.3	16.1
75-95	18.6	288.6	68	68.8	17.9
95-100	2.9	36.0	85	76.0	21.9
Total	57.9	1465.0	42	64.7 (37.5)[a]	13.6

[a] % of total ^{14}C in supernatant.

Carbaryl In Soils

Decomposition of carbaryl (1-naphthyl N-methylcarbamate) by a soil bacterium was reported by Tewfik and Hamdi (1969). Four unidentified metabolites were produced, one which was possibly salicylic acid. In Bacteriological Proceedings of 1970, Bollag and Liu described biodegradation of carbaryl by soil microbes. All microorganisms isolated hydrolyzed carbaryl to 1-naphthol. One-naphthol was metabolized rapidly by Fusarium solani, degraded gradually by a Gram (-) coccus and accumulated in a Gram (+) rod. Disappearances of ring-labeled 1-naphthol and carbaryl labeled on the carbamate moiety were compared with F. solani and the coccus singly and mixed. The fungus effected an almost complete loss of radioactivity from the carbaryl incubation, but only 20% of the radioactivity in the 1-naphthol preparations. The coccus attacked carbaryl more effectively than F. solani. The mixture degraded both carbaryl and 1-naphthol, suggesting that complete biodegradation was a result of combined growth.

More recent work on the metabolism of carbaryl by soil fungi showed that several species of the genera Aspergillus, Fusarium, Gliocladium, Mucor, Penicillium and Rhizopus produced varying amounts of 1-naphthyl N-hydroxymethylcarbamate and the 4- and 5-hydroxylated metabolites (Bollag and Liu, 1971). Gliocladium roseum was selected for further study and the three metabolites firmly identified by ultraviolet, infrared and mass spectroscopy. Aspergillus terreus produced minute amounts of what were tentatively identified as the ring-hydroxylated metabolites of the carbamate insecticide (Liu and Bollag, 1971b). Two metabolites, the formation of which followed the same pattern over a period of 8 days of incubation in yeast extract-nutrient broth, were identified as 1-naphthyl N-hydroxymethylcarbamate and 1-naphthyl carbamate. A. terreus grew on media containing either of the two metabolites or 1-naphthol. The authors postulated that degradation proceeds from 1-naphthyl N-hydroxymethyl carbamate to 1-naphthyl carbamate to 1-naphthol. Since the medium controls contained more 1-naphthol than did A. terreus cultures, the fungus was assumed to actively degrade 1-naphthol (Liu and Bollag, 1971a).

In our study, radiolabeled carbaryl was added to non-sterile and autoclaved soils and to culture media inoculated either with soil suspensions or with pure cultures of microorganisms selectively isolated from soil. Polar water-soluble metabolites of radiolabeled carbaryl produced in bean plants were also added to soil. After incubation, the soils and culture media were assayed to determine the fate of the radiolabeled material. The microorganisms which were capable of attacking the carbaryl molecule to a demonstrable degree under the experimental conditions employed were identified.

Methods

Soils - Three samples of Maury soil of the type common to the Inner Bluegrass region but with different histories of pesticide treatment were employed in the soil studies. These soils have been designated as Untreated, Carbaryl-treated, and Mixed Pesticide-treated soils. The Untreated and Carbaryl-treated soils were taken from neighboring plots in the same tobacco field. They were similar as to color, consistency and amount of organic material. The former had received no recorded pesticide treatment of any kind, while the latter had received 4 lb/A Sevin ^{R} granules six months prior to the sampling date. This level of treatment approximates 4 ppm if uniform distribution to a depth of 6 2/3 inches is assumed (Bollen, 1961). The Mixed Pesticide-treated soil was taken from a home orchard floor which had been subjected for fifteen years to various treatments including runoff from foliar sprays of insecticides (including carbaryl) and fungicides applied to the trees, soil treatments, and herbicide drift. This soil was observed to contain more organic material than the other two.

The samples were taken from the upper six inches of topsoil and contained 18-22 percent moisture when brought into the laboratory. They were stored in heavy plastic bags, tightly closed, at room temperature until used in the soil studies.

Chemicals - The radiolabeled compound employed was carbaryl-naphthyl-1-^{14}C of specific activity 0.6 mc/mM or 6.6 x 10^3 dpm/ug. In addition, radiolabeled polar water-soluble metabolites of carbaryl prepared from bean plants were used. Polar water-soluble metabolites of carbaryl (PMC) were prepared by injecting carbaryl-1-naphthyl-1-^{14}C into young bean plants and, after a suitable interval, extracting the water-soluble metabolites produced in the plants. Eleven-day old Cranberry bush bean seedlings were injected with carbaryl-1-naphthyl-1-^{14}C at the rate of 50 ug/plant in 50 ul of a 3:1 mixture of water and acetone. The solution was injected into the stem just above the soil line using a 50 ul syringe. Prior to the injection, a small pinhole was made in the stem about 2 inches above the injection site to permit escape of air. Following injection, the holes were closed with silicone stopcock grease.

Dorough and Wiggins (1969) established optimal harvest time for production of water-soluble metabolites and their precise location in Contender variety beans. On the basis of their findings, a single Cranberry bush plant was harvested 9 days after injection and the epicot leaves extracted. Since water-soluble metabolites were found in amounts comparable to those that had been found in Contender variety, the plants were harvested at 10 days after injection. The epicot leaves were removed and stored at -10°C until extracted. A pair of epicot leaves from a plant was homogenized 5 min at medium-high speed in a Vir-Tis 45 Homogenizer in 100 ml of 90 percent acetone in water. The homogenate was filtered with suction through Whatman no.1 filter paper, and the marc rinsed with acetone. The filtrates from three pairs of leaves were combined, then fractionated into water-solubles and organosolubles by shaking in a 1000-ml separatory funnel with an equal volume of chloroform. The organic layer was washed twice with 25 ml of water, then the aqueous layers were combined and washed twice with an equivalent volume of chloroform. The foregoing procedure was the same as that of Wiggins and Weiden (1969) except that they used five chloroform rinses. The aqueous layer containing the PMC was concentrated by lyophilization.

The nature of the organic moieties (aglycones) contained in the PMC was determined by enzymatic hydrolysis, extraction of the hydrolysate, and tlc analysis of the extract. In the hydrolysis procedure used a sample containing PMC in amounts up to 100,000 dpm was adjusted to a volume of 2.2 ml in water and placed in a 25-ml Erlenmeyer flask. To this was added 20 ul of glusulase, 0.08 ml of isopropanol, 4.0 ml of citrate-phosphate buffer at pH 5.0, and 1 drop of toluene. The mixture was incubated in a water bath with shaking for one hour at 37-38°C, after which it was transferred to a 60-ml separatory funnel and extracted three times with 25-ml amounts of methylene chloride. The combined extracts were dried with sodium sulfate, filtered through Whatman no. 1 filter paper and evaporated to a volume suitable for tlc.

For preparation of Miles (Miles et al., 1969) carbaryl agar, the salts and agar were dissolved in distilled water and the pH was adjusted to 7.0 with 1N potassium hydroxide. The medium was then autoclaved and cooled to 45-50°C. Carbaryl dissolved in ethanol was then added in amounts calculated to give 1 ppm and 1 percent of carbaryl and ethanol, respectively, in the Miles carbaryl agar. Twenty-five ml amounts were then poured into sterile petri plates. Carbaryl-treated soil was suspended in distilled water in different proportions and 1 ml of each spread over the surface of a plate. The plates were incubated at 25°C. As isolated colonies of fungi appeared, they were transferred to fresh plates, either by center transplant or by successive streaks 2 cm apart across the plate. Bacterial colonies were transferred by conventional streakouts to plates or slants of Miles carbaryl agar. As in the silica gel procedure, repeated transfers were made when necessary for purification.

Soil studies - Radiolabeled carbaryl, 1-naphthol and plant metabolites were added to soil samples along with autoclaved soil samples for controls. The resultant preparations were incubated under controlled conditions. Immediately and after various periods of incubation portions of the soil preparations were removed for analysis of the radioactive components. Total radioactivity was determined by direct count or by assaying trapped carbon dioxide following combustion of the soil or by making an acetone-water extract of the soil, fractionating the extract with chloroform into water-soluble and organosoluble portions and determining the radioactivity of each extract, as well as that of the residue remaining after extraction. The organosoluble portions were subjected to tlc analysis for separation and identification of metabolites formed from carbaryl and from PMC. The production and nature of volatile metabolites produced during incubation was investigated by the use of trapping systems.

In making soil preparations, the soil was sieved through hardware cloth of 1/4 inch mesh, and moisture was determined by drying duplicate samples at 65°C for 48 hours. An aliquot of soil comprising about 10 percent of the total amount to be incubated was oven-dried. The appropriate amounts of carbaryl or 1-naphthol dissolved in acetone or of PMC in water (filter-sterilized if to be used in autoclaved soil preparations) were added to the oven-dried soil. When acetone was used, the soil and acetone were stirred constantly until the acetone odor was no longer detectable. The radioactive soil was then combined with the remaining soil to be incubated, distilled water added to provide 17-20 percent moisture, and the whole soil mass mixed thoroughly. Zero-time samples taken immediately by picking up randomly selected small amounts of soil and radioassaying by combustion established that carbaryl was distributed in a reasonably homogeneous fashion throughout the soil. The soil preparations were incubated in cotton-capped Erlenmeyer flasks at 27°C and 85 percent relative humidity in an environmental chamber. Samples for analysis were removed at designated intervals and stored at -10° until examined.

A 10-g soil sample was placed in 50 ml of a 5:1 acetone-water solution and shaken by hand for 5 min in a tightly-stoppered 250-ml Erlenmeyer flask. The suspension was filtered with suction through 2 layers of Whatman no. 1 filter paper. The solids were rinsed with a 5:1 acetone-water, air-dried, weighed, and saved for radioassay. The filtrate was partitioned with 60 ml of chloroform and the water layer re-extracted with 25 ml of chloroform. The organosoluble layer was radioassayed, dried with sodium sulfate, evaporated under reduced pressure to a volume of 4 or 5 ml, and further concentrated under a gentle air stream to a volume suitable for tlc.

For the soil preparations with plant metabolites added the procedures of extraction and fractionation were modified. Ten grams of soil were placed with 50 ml of 1:1 acetone-water in a 250-ml flask and shaken vigorously by hand for 5 min. By this procedure 70 percent of the PMC were recovered from zero-time soil. The suspensions were filtered and the soil residues dried for radioassay. The filtrates were partitioned with volumes of chloroform equivalent to those of acetone in the filtrates. Both aqueous and organic extracts were evaporated under reduced pressure, and further concentrated under a gentle air stream to volumes suitable for tlc.

Total radioactivity in the soil preparations was determined in two ways: (1) by radioassay of an aliquot of soil following combustion in either Parr oxygen bombs or the Beckman Biological Materials Oxidizer (BMO), or directly; (2) in the cases where soil was subjected to extraction and fractionation procedures, by addition of the values for radioactivity in the organosoluble extract, the water-soluble layer, and the extracted residue.

Liquid extracts and concentrates were radioassayed by direct count: a suitable aliquot was pipetted directly into a counting vial, 10 ml of scintillation fluid were added, the vial capped, shaken, and placed in the spectrometer to be counted. Aliquots consisted of 0.2 to 0.5 ml for organosoluble extracts, 0.02 ml for organic concentrates, and 0.2 ml for water-soluble materials.

Chromatography and identification - For separation of carbaryl from its metabolites, and the concentrated organosoluble extract was first applied as a band to a thin layer chromatogram which was developed one-dimensionally. The chromatograms were made on either Silica Gel G 0.25 to 0.5 mm thick on a 20 x 20 cm glass plate or ChromAR 500 thin-layer sheets (Mallinckrodt Chemical Works). The solvent systems for one-dimensional chromatography were ether-hexane combinations in proportions varying from 2:1 to 9:1. A short band of carbaryl was applied to each chromatogram as a standard. Radioautographs were prepared by exposing the developed tlc plates to x-ray film at -10°C for a minimum of seven days. If unknown carbaryl metabolites appeared as bands on the radioautographs, the bands were radioassayed in one of two ways depending on the apparent intensity of the band. If the band represented a metabolite of sufficient quantity for further chromatographic analysis, the metabolite was extracted from the chromatogram with 1:1 acetone-methanol or with acetone alone. An aliquot of the extract was then counted.

PMC as prepared from bean plants were separated directly by applying a small amount of the aqueous concentrate as a band to a thin layer plate of Silica Gel F-254 0.5 mm thick and developing the chromatogram one-dimensionally in 65:25:4 chloroform-methanol water (Mumma et al., 1971). After PMC were extracted from incubated soil and the extract was fractionated, direct separation of the metabolites in the aqueous layer was not possible because of interfering substances present therein. The organosoluble fraction of the extract was dried with sodium sulfate and concentrated to tlc volume. The concentrate was applied as a spot to Silica Gel F-254 and the chromatogram developed two-dimensionally, the solvent systems being 2:1 ether-hexane followed by 7:2 methylene chloride-ethyl acetate.

R_f values were computed for unknown carbaryl metabolites appearing as bands on chromatograms and these were compared to R_f values of known carbaryl metabolites in the same solvent system. If present in sufficient quantity, the unknown metabolite was extracted as described above and the extract concentrated to tlc voluem. It was then compared by tlc with one or more known metabolite standards having similar R_f values. Non-radioactive carbaryl standards were visualized by examination of the chromatograms under UV light and/or by spraying the chromatograms with 15 percent sodium hydroxide followed by exposure to iodine vapors in a tightly closed glass chromatography tank.

Determination of volatile metabolites - Soil preparations containing carbaryl-1-naphthyl-1-^{14}C, 1-naphthol-1-^{14}C, and, in one experiment, with carbaryl-carbonyl-^{14}C, were incubated in systems for the determination of the evolution of volatile metabolites such as 1-naphthol and carbon dioxide. The 1-naphthol soil preparations were included in the studies on the basis of the simultaneous adaptation theory of Stanier (1947). According to this theory, if a microorganism can utilize a compound as a carbon source and metabolize it through a particular pathway, it possesses all the enzymes of the pathway and thus should be able to attack any intermediate in the pathway and utilize it likewise. If the soil preparation metabolizes carbaryl through hydrolysis, it also should be able to attack the intermediate 1-naphthol.

For these studies, soil preparations were held at laboratory temperature and ambient humidity (24°C and 60% R.H.). Using this type of system, Carbaryl-treated soil, both non-sterile and autoclaved, was incubated for three days with carbaryl-1-^{14}C and also with 1-naphthol-^{14}C. At intervals, air was bubbled through the system to drive volatile metabolites through the gas dispersant tubes into 20 ml of the organic trap solution. In a corollary test, Carbaryl-treated soil was incubated with carbaryl-1-^{14}C in a system having four 250-ml flasks set up in series. The first two flasks contained saturated barium hydroxide to clear carbon dioxide from the air before it entered the third flask containing the soil preparation. The fourth flask contained barium hydroxide to trap the radioactive carbon dioxide arising from the soil preparation. In both tests, zero-time and terminal samples were removed for extraction and radioassay. When barium hydroxide served as the trap solution, barium carbonate formed during incubation was collected by filtration washed with distilled water, air-dried on the filter paper and radioassayed following combustion in the Parr bomb. Efficiency of combustion of barium carbonate was ascertained by combusting a known amount of carbaryl-^{14}C in the presence of non-radioactive barium carbonate on filter paper. Seventy five percent recovery was obtained. Values obtained for radioactive samples were adjusted accordingly. For detection of water-soluble materials such as the barium salt of 1-naphthol, the filtrate was radioassayed by direct count.

In another study, an acetone trap preceded the outlet barium hydroxide trap. Volatile organic metabolites if present would be detected in the acetone trap, and carbon dioxide would pass through the acetone to be trapped by barium hydroxide. The Carbaryl-treated soil preparation was incubated with carbaryl-1-^{14}C for 13 days. During the day, an air stream was passed through the system at 20 ml/min. The trap solutions were removed, refrigerated, and replaced with fresh solutions three times during the course of the study. Aliquots of acetone was evaporated under reduced pressure and aliquots again counted to determine low levels of radioactivity. Barium carbonate was assayed as described above. Total radioactivity in the zero-time and 13-day soil samples was determined following combustion in the Biological Materials Oxidizer.

In the final study of the series, duplicate 250-ml Erlenmeyer flasks of Carbaryl-treated and Untreated soils were incubated for 56 days with each of the following labeled materials: carbaryl-1-naphthyl-1-^{14}C, 1-naphthol-1-^{14}C and carbaryl-carbonyl-^{14}C. The 12 flasks were connected through a manifold assembly to a common inlet air stream pre-cleared of carbon dioxide by being bubbled through the organic trap solution. Outlet tubes from the flasks were attached to gas dispersant tubes immersed in 20 ml amounts of the same trap solution. Air was passed through the system during the day at a flow rate sufficient to keep the slowest unit bubbling continuously. At intervals, the trap solutions were removed, aliquots radioassayed, and the traps filled with fresh solutions.

Culture media studies - Culture media containing carbaryl-1-naphthyl-1-^{14}C were inoculated with suspensions of Carbaryl-treated and Mixed Pesticide-treated soils and with pure cultures of bacteria and fungi. After incubation, the media were extracted and assayed for carbaryl and its metabolites.

Results and Discussion

Soil studies - In a 120-day study carried out to determine the fate of carbaryl in non-sterile and autoclaved Untreated, Carbaryl-treated and Mixed Pesticide-treated soils, carbaryl-1-^{14}C was added at 7.0-10.0 ppm. A striking difference was seen in the rates of dissipation of carbaryl from the non-autoclaved soils with three histories of pesticide treatment (Table 27). Apparently more than 60 percent of the radioactivity had disappeared completely after four days in the Carbaryl-treated soil. At the same time the other soils had lost less than 7 percent. During the first 14 days of incubation the radioactivity disappeared from Untreated and Mixed Pesticide-treated soils at about the same rate. Beyond that time, dissipation proceeded more slowly in the latter. After 120 days, the Untreated, Carbaryl-treated and Mixed Pesticide-treated soils still retained 23, 16 and 30 percent, respectively, of the initial radiocarbon.

Autoclaved soil did not retain total sterility throughout 120 days. Fungal development was detected in 14 days in Untreated and Carbaryl-treated soils, and at 120 days in Mixed Pesticide-treated soil. However, in any given soil, radioactivity disappeared much faster from non-autoclaved than from autoclaved soil. This fact suggests that increased dissipation rates in non-autoclaved soil reflected biological action of soil microorganisms.

Organosoluble radiocarbon in the zero-time soil ranged from 90 to 97 percent and represented essentially the extraction efficiency of the acetone procedure (Table 28). More than 90 percent of the total organosoluble radiocarbon added to Carbaryl-treated soil was lost after 4 days. This pattern was not seen in the other two soils, where a comparable loss was noted only after 42 days. Tlc analysis of organosoluble extracts from all the timed samples in these soils revealed that the radioactivity was composed entirely of carbaryl-1-^{14}C. Water-soluble metabolites remained rather low (less than 2 percent) throughout the study. Unextracted ^{14}C-residues increased in quantity initially but later decreased in the Untreated and Carbaryl-treated soils.

In order to determine more exactly the time of early dissipation of carbaryl from Carbaryl-treated soil, a corollary 7-day study was conducted, using non-sterile soils in conditions identical to those of the 120-day study. Analysis of samples removed at 0., 1, 2, 3, 4 and 7 days confirmed the difference in dissipation rates of radioactivity from the three soils found in the 120-day study. The most rapid loss of label (44 percent) occurred between the first and second day.

The fate of polar water-soluble metabolites of carbaryl (PMC) in soil was investigated in 2 studies. In the first, PMC were added at 2650 dpm/g to non-autoclaved and autoclaved Carbaryl-treated soils, which were then incubated at laboratory temperature and ambient humidity (approximately 24°C, 60% R.H.) for 14 days.

After 7 days of incubation with the non-autoclaved soil, about 50 per cent of the initially added PMC had been dissipated (Table 29). No further loss occurred in the period between 7 and 14 days. In autoclaved soil, no such dissipation occurred. In the second study, PMC were added at 9074 dpm/g to non-autoclaved Untreated, Carbaryl-treated and Mixed Pesticide-treated soils, which were then incubated for 120 days in the environmental chamber. After the first 14 days, 55, 64 and 50 percent of the radiocarbon remained in the Untreated, Carbaryl-treated and Mixed Pesticide-treated soils, respectively. Little further loss occurred from 14 to 120 days (Table 30). This indicated that the biological attack of soil microorganisms on PMC occurred early in the incubation period, and that residues left after the initial attack were generally untouched by biological action thereafter.

After prolonged incubation the PMC residues were largely unextractable by the procedures available. Only 15 percent of the total PMC still present were recovered in the extract. This finding necessitated pooling of the samples in order to have enough material in the extracts for characterization of the aglycones. Samples taken between 7 and 28 days, and those taken between 42 and 120 days of incubation were each treated as one sample for extraction purposes.

Before incubation with soil, the aglycone moieties resulting from enzymatic hydrolysis of the PMC were identified by cochromatography with known standards as 5,6-dihydro-5,6-dihydroxycarbaryl, hydroxymethyl carbaryl, 7-hydroxycarbaryl, 4-hydroxycarbaryl (or 6-hydroxycarbaryl, the two being inseparable in the solvent systems used), 5-hydroxycarbaryl, carbaryl and 1-naphthol. Small amounts of three aglycones appeared in the organic fractions of the extracts of Mixed Pesticide-treated soil, and the same three plus an additional one in the organic fractions of the extracts of Untreated and Carbaryl-treated soils. The positions to which these four aglycones migrated when the organic concentrate was applied to a two-dimensional chromatogram indicated that they were different than those prepared by enzymatic hydrolysis of the PMC before incubation. Amounts of the four aglycones recovered were too small to make positive identifications; however, it was noted that one metabolite migrated to the region of the chromatogram where naphthalenediols are generally found.

When Carbaryl-treated soil preparations incubated with carbaryl-1-^{14}C and 1-naphthol-1-^{14}C were connected to trap solutions, carbon-14 dioxide was trapped (Table 31). In 3 days, 18.3 percent and 3.1 percent of the total radiocarbon from carbaryl and 1-naphthol, respectively, appeared in the organic trap solution. Comparable total percentages of ^{14}C equivalents were recovered from carbaryl and 1-naphthol. With the latter, however, less radiocarbon was found in organosolubles and carbon dioxide and more in water-solubles and unextractables than was the case with carbaryl. No carbon dioxide was ever detected in trap solutions attached to autoclaved soil incubated for 3 days with either carbaryl-1-^{14}C or 1-naphthol-1-^{14}C. Radioassay of the soil after 3 days of incubation indicated that the labeled materials added initially had not dissipated at all. Another flask culture, incubated with carbaryl-1-^{14}C for 7 days attached to a barium hydroxide trap gave off 22.6 percent of the initial label as carbon dioxide. In these studies, carbaryl was dissipated less rapidly when incubated at laboratory temperature and ambient humidity that it had been in the earlier persistence studies carried out in the environmental chamber.

When Carbaryl-treated soil was incubated for 13 days with carbaryl-1-^{14}C with acetone and barium hydroxide traps attached in series, less than 1 percent of the radiocarbon was accumulated in the acetone traps as volatile materials other than carbon dioxide. This was a negligible amount, compared to 30 percent trapped as carbon dioxide in the barium hydroxide traps (Table 32). No radioactivity was ever detected in direct counts of aliquots of filtrates from the barium hydroxide trap solutions. This precluded the presence of water-soluble materials such as barium salts of organic metabolites. It was thus established that the volatile radioactive materials arising from soils incubated with carbaryl-1-^{14}C were essentially carbon dioxide.

The last soil study conducted was designed to compare carbon dioxide production from Untreated and Carbaryl-treated soils incubated with ring-labeled carbaryl, carbonyl-labeled carbaryl, 1-naphthol-1-^{14}C (Table 33). It was observed that consistently throughout the incubation period with any radiocarbon source, more carbon dioxide was trapped from Carbaryl-treated than from Untreated soil. In the Untreated soil, the highest percentage of carbon dioxide arose from 1-naphthol-1-^{14}C, especially in the early days of the study. In the Carbaryl-treated soil the same effect was noted in the first three days of incubation; comparison of the total carbon dioxide liberated, however, shows that comparable percentages were lost from 1-naphthol-1-^{14}C and carbonyl-labeled carbaryl.

At the time this study was conducted, nearly 1 1/2 years had elapsed since the soils were brought into the laboratory and the first persistence study was initiated. During the entire 56-day incubation period, 7 percent of the initial radiocarbon was trapped from 1-naphthol-1-^{14}C and carbonyl-labeled carbaryl and 4 percent from ring-labeled carbaryl in the Carbaryl-treated soil. Decreased degradation of carbaryl is believed to have resulted from the fact that the soil had been stored in the laboratory for 1 1/2 years. Bartha

(1.71) found that air-drying of soil severely reduced its capacity to metabolize propanil.

Culture media studies - When AA medium with 3.3 ppm carbaryl-1-^{14}C and 1 percent glucose was inoculated with a suspension of Mixed Pesticide-treated soil and incubated at 30°C with continuous shaking for 96 hours, 73 percent of the carbaryl was recovered unchanged from the medium, as compared to 77 percent from the medium control (Table 34). In the soil cultures, organosoluble, water-soluble and unextractable radioactivity increased with time, while in the medium control only the first two fractions increased. Water-soluble radioactivity, as used here, included both radioactivity found in the aqueous medium after extraction and radioactive material which did not migrate away from the origin of the thin-layer chromatograms made from the organic concentrates. In the soil cultures, the percentages of radioactive water-solubles given in Table 34 represent predominantly material found in the aqueous layer rather than on the chromatograms, while in the medium control the reverse was true.

The nature of the organosolubles in the soil cultures differed from that of those found in the medium control. In the cultures, small amounts of one organosoluble metabolite appeared after 24 hours of incubation and a second appeared after 48 hours. By 96 hours the second had accumulated to a greater extent than the first. This suggests that the first metabolite may have served as a precursor for the second in the degradation pathway of carbaryl. The quantity of the first metabolite was not sufficient for extraction and two-dimensional chromatography. Its R_f value one-dimensionally resembled that of hydroxymethylcarbaryl. The second metabolite had an R_f value similar to those of the hydroxylated carbaryl metabolites in one-dimensional chromatograms, but did not cochromatograph with any of them two-dimensionally. Other than the parent compound, the only organosoluble metabolite found in the medium control was 1-naphthol, which did not appear in any of the cultures. Two possible explanations may be suggested. Carbaryl is subject to chemical hydrolysis at alkaline pH and to photolysis; in cultures grown on glucose, acid production may have prevented chemical hydrolysis and/or turbidity in the incubation flasks may have hindered photolysis by interfering with transmission of light. It is also possible that free 1-naphthol was produced in the cultures and conjugated too rapidly into water-soluble metabolites to be detected in the organosoluble fraction. When Liu and Bollag (1971a) found more 1-naphthol in medium controls than in *Aspergillus terreus* cultures incubated with carbaryl, they assumed that *A. terreus* was actively degrading the 1-naphthol. Recovery after incubation of more than 90 percent of the initial radioactivity indicated that in these cultures no more than negligible amounts of radioactive carbon dioxide escaped.

In studies using Carbaryl-treated soil suspensions and bacterial and fungal isolates as inocula, incubation proceeded for 14 days at laboratory temperatures and no attempt was made to follow the time course of metabolite production. The results in Table 35, as well as those in Tables 36 and 37, represent averages of the results of the designated number of duplicate experiments. When a suspension of non-sterile Carbaryl-treated soil was used as the inoculum in Miles medium, only 19 percent of the radioactivity could be accounted for by unchanged carbaryl; other organosolubles accounted for less than 1 percent, water-solubles for 30 percent, and unextractables 23 percent, of the original radiocarbon (Table 35). In the medium inoculated with autoclaved Carbaryl-treated soil, 71 percent of the initial label was in unchanged carbaryl, 3 percent in other organosolubles, 9 percent in water-solubles and 6 percent in unextractables, with a total recovery of 89 percent. In the medium control, 92 percent of the initial radiocarbon was recovered: 76 percent as the parent compound, 6 percent as other organosolubles, 8 percent as water-solubles, and 2 percent as unextractable radiocarbon that remained on the filter paper during the filtration step in the extraction procedure. The drastically reduced percentage of unchanged carbaryl when the medium was inoculated with the non-sterile soil indicated biological action on the carbaryl molecule. However, except for traces of 1-naphthol in one flask, organosoluble metabolites were absent from media inoculated with the non-sterile soil. Dissipation of 28 percent of the total label from such media compared to only 11 percent from media inoculated with autoclaved soil could well indicate some loss from the former as carbon dioxide. No attempt was made to trap volatile radiocarbon in the culture medium studies.

The fungal cultures gave some evidence of attack on the carbaryl molecule. The percentages of unchanged carbaryl found with fungal isolates, except for *Penicillium implicatum* and SF-10, were similar to those found with autoclaved soil and in the medium control (Table 35). *P. implicatum* showed an increase in total water solubles and SF-10, which had been identified tentatively as a non-sporulating variant of *Aspergillus terreus*, an increase in total organosolubles and unextractables. The percentages of total radioactivity recovered after incubation with fungal isolates indicated little if any loss of carbon dioxide. Although some of the fungal strains produced no demonstrable change in total radioactivity, all of them produced some metabolites.

SF-10 and *P. implicatum* produced a metabolite which was identified by two-dimensional cochromatography as hydroxymethylcarbaryl. SF-10 and the three *Fusarium* species produced an organosoluble metabolite that had an R_f value in 2:1 ether-hexane similar to those of the hydroxylated metabolites of carbaryl that migrate beyond hydroxymethylcarbaryl in this system. However, extensive efforts at cochromatography in two-dimensional systems established that the metabolite was neither one of the common hydroxylated compounds nor 1-naphthylcarbamate. *P. implicatum* also formed a metabolite which had an R_f value in 2:1 ether-hexane similar to those of 1-naphthylcarbamate and 4-methoxycarbaryl.

In two-dimensional chromatography it formed 2 spots which approximated the spots of carbaryl and 1-naphthol. Penicillium lilacinum and and Aspergillus elegans produced very small amounts of a metabolite having an R_f value in 2:1 ether-hexane similar to that of 5,6-dihydro-5,6-dihydroxycarbaryl.

When three strains of bacteria isolated from Carbaryl-treated soil were incubated with carbaryl in Miles medium no evidence of any action on the carbaryl molecule was found (Table 36). These strains proved incapable of growth in nutrient broth and were dropped from further consideration.

The bacterial species isolated from Mixed Pesticide-treated soil were transferred several times on trypticase soy agar slants before incubation with carbaryl-1-^{14}C. These species attacked carbaryl effectively when cultured in nutrient broth (Table 37). After 14 days of incubation, with all except Arthrobacter tumescens, less than 10 percent of the initial carbaryl was recovered as the parent compound. Pseudomonas acidovorans, Nocardia flava and Arthrobacter sp. converted carbaryl largely to water-soluble metabolites, while Xanthomonas sp. and Bacillus sphaericus produced relatively more organosoluble metabolites. B. sphaericus also grew on Miles medium but degraded carbaryl less effectively (Table 36) than in nutrient broth. In considering the nutrient broth culture results it should be mentioned that in the medium control only 60 percent of the initial carbaryl-1-^{14}C was recovered as parent compound, and 24 percent of the radioactivity was found in the water layer. This was not a case of lesser extraction efficiency of the acetonitrile-chloroform procedure on nutrient broth; when carbaryl-1-^{14}C was added to medium and re-extracted without incubation, 93 percent and 95 percent were recovered from Miles medium and nutrient broth, respectively. Hence, the fundamental conditions in nutrient broth brought about greater degradation of carbaryl than those in Miles medium.

Two organosoluble metabolites with R_f values in 2:1 ether-hexane greater than that of the parent compound appeared in extracts of cultures of *B. sphaericus* and *Xanthomonas* sp. The two metabolites did not separate completely in one dimension and did not migrate cleanly in the two-dimensional system generally used in these studies for identification of metabolites (2:1 ether-hexane followed by 7:2 methylene chloride-ethyl acetate). The best separation was achieved two-dimensionally on Silica Gel F-254 with 5:1 hexane-ether used in both dimensions. One of the metabolites was identified as 1-naphthol while the other migrated beyond 1-naphthol in non-polar solvent systems and was not identified. Traces of a third metabolite were produced by *Arthrobacter* sp., *Nocardia flava* and *Xanthomonas* sp. Its R_f value in 2:1 ether-hexane was the same as one of the fungal metabolites which was not identified.

TABLE 27. RADIOACTIVITY OF SOILS FORTIFIED WITH CARBARYL-1-$^{14}C^{a}$ AND INCUBATED AT 27°C, 85% R.H.

Days of Incubation	Soils					
	Untreated		Carbaryl-treated		Mixed Pesticide-treated	
	NA	A	NA	A	NA	A
4	93.5	-	38.5	-	94.6	-
7	89.0	80.1	33.7	84.1	102.2	109.8
14	60.8	76.6	24.8	58.7	62.6	90.0
28	33.7	75.4	22.1	67.8	53.6	97.0
42	30.2	75.9	22.7	63.0	38.4	113.1
120	23.1	46.7	16.0	62.8	29.9	60.1

[a] 10 ppm in non-autoclaved and 7 ppm in autoclaved soils.

[b] Percent of initial radioactivity

A = autoclaved soil; NA - non-autoclaved soil.

TABLE 28. RADIOACTIVITY IN ORGANOSOLUBLES (OS), WATER SOLUBLES (WS) AND UNEXTRACTABLES (U) IN SOILS FORTIFIED WITH CARBARYL-1-^{14}C AND INCUBATED AT 27°C, 85% R.H.

Days	Soils								
of	Untreated			Carbaryl-Treated			Mixed-Pesticide treated		
Incubation	OS	WS	U	OS	WS	U	OS	WS	U
0	94.2[a]	0.3	5.5	89.7	0.6	9.7	96.8	0.2	3.0
4	67.9	0.5	21.1	3.6	1.0	23.6	81.2	0.4	15.4
7	61.0	0.6	22.5	1.7	1.0	20.8	67.4	0.5	17.1
14	34.1	1.0	27.0	1.7	1.1	20.2	50.5	0.7	22.0
28	14.6	1.2	23.3	1.0	1.5	13.5	20.7	0.7	28.6
42	3.6	1.5	24.6	0.7	1.1	16.6	7.4	1.5	30.5
120	1.2	1.7	17.0	0.6	0.9	12.6	1.5	2 .0	26.9

[a] Percent of initial radioactivity.

TABLE 29. RADIOACTIVITY OF CARBARYL-TREATED SOIL FORTIFIED WITH POLAR WATER-SOLUBLE METABOLITES OF CARBARYL-^{14}C[a] AND INCUBATED IN THE LABORATORY

Days of incubation	Fraction	Soils	
		Non-autoclaved	Autoclaved
7	Extractables	11.7[b]	74.3
	Unextractables	39.7	32.3
	Total	51.4	106.7
14	Extractables	6.7	74.2
	Unextractables	50.5	24.9
	Total	57.2	99.1

[a] Level of radioactivity in soil 2,650 dpm/g.

[b] Percent of initial radioactivity.

TABLE 30. RADIOACTIVITY OF SOILS FORTIFIED WITH POLAR WATER-SOLUBLE METABOLITES OF CARBARYL-^{14}C[a] AND INCUBATED AT 27°C, 85% R.H.

	Soils		
Days of Incubation	Untreated	Carbaryl-treated	Mixed Pesticide-treated
0	100.0[b]	100.0	100.0
7	63.7	64.8	67.2
14	54.9	63.9	49.7
28	57.9	56.3	71.2
42	60.5	60.6	61.8
56	62.6	66.2	44.2
84	63.2	55.1	58.7
120	54.2	53.6	57.4

[a] Level of radioactivity in soil 9,074 dpm/g

[b] Percent of initial radioactivity.

TABLE 31. FATE OF CARBARYL-NAPHTHYL-1-^{14}C AND 1-NAPHTHOL-^{14}C IN CARBARYL-TREATED SOIL HELD UNDER LABORATORY CONDITIONS

Substance added	Days of Incubation	Percent of added radioactivity present as				
		Organo-solubles	Water solubles	Unex-tractables	Carbon Dioxide	Total Recovery
Carbaryl-^{14}C	3[a]	45.6	0.4	25.1	18.3	89.4
1-Naphthol-^{14}C[a]	3[a]	24.2	16.9	48.4	3.1	92.6
Carbaryl-^{14}C	7[b]	35.3	0.8	17.1	22.6	75.8

[a] Air passed from soil preparation through trap solution consisting of a 2 to 1 mixture of 2-methoxyethanol and 2-aminoethanol.

[b] Air passed from soil preparation through barium hydroxide trap.

TABLE 32. RADIOACTIVITY OF ACETONE AND BARIUM HYDROXIDE TRAP SOLUTIONS AFTER INCUBATION OF CARBARYL-TREATED SOIL PREPARATIONS WITH CARBARYL-1-NAPHTHYL-1-^{14}C ADDED[a]

Days of Incubation	Cumulative radioactivity expressed as percent of initial radioactivity	
	Acetone Trap	Barium Hydroxide Trap
0-3	0.1	10.3
4-6	0.2	21.7
7-10	0.3	26.6
11-13	0.4	29.5

[a] Carbaryl-^{14}C added to soil at the 1.5 ppm level and air passed from the preparation through acetone and then barium hydroxide solution.

TABLE 33. CARBON-14 DIOXIDE LIBERATED FROM CARBARYL-^{14}C AND 1-NAPHTHOL-^{14}C DURING INCUBATION WITH SOIL

Material	Untreated soil				Carbaryl-treated soil			
	Days of incubation				Days of incubation			
Incubated[a]	0-3	4-7	8-56	Total	0-3	4-7	8-56	Total
Carbaryl-1-naphthyl-1-^{14}C	0.2[b]	0.1	0.2	0.5	2.5	0.5	0.6	3.6
Carbaryl-carbonyl-^{14}C	0.8	0.3	0.7	1.9	2.9	1.8	2.1	6.8
1-Naphthol-1-^{14}C	3.7	0.6	0.9	5.2	4.7	0.8	1.1	6.6

[a] Added at 1.5 ppm.

[b] Percent of initial radioactivity.

TABLE 34. RADIOACTIVITY OF CULTURE MEDIUM WITH CARBARYL-$^{14}C^{a}$ INOCULATED WITH SUSPENSION OF MIXED PESTICIDE-TREATED SOIL

	Hours of incubation			
With soil inoculum:	12	24	48	96
Carbaryl	100.9[b]	88.4	90.1	73.2
Organosolubles	0	0	0.5	2.3
Water solubles	0.5	1.6	4.0	8.8
Unextractables	0	0	0.6	6.7
Total	101.4	90.0	95.2	91.0
Uninoculated Medium Control:				
Carbaryl	83.2	83.2	86.0	76.8
Organosolubles	0.8	2.4	3.7	5.5
Water solubles	0.6	0.7	1.7	9.1
Unextractables	0	0	0	0
Total	84.6	86.3	91.4	91.4

[a] Carbaryl-^{14}C at 3.3 ppm with 1% glucose.

[b] Percent of initial radioactivity.

TABLE 35. RADIOACTIVITY OF MILES MEDIUM WITH CARBARYL-1-$^{14}C^{a}$ INOCULATED WITH CARBARYL-TREATED SOIL SUSPENSIONS OR FUNGAL ISOLATES AND INCUBATED 14 DAYS

	Radioactivity as				
Inoculum	Carbaryl	Water-solubles	Organo-solubles	Unex-tractables	Total
Medium control	75.5[b]	7.6	6.5	2.4	92.0
Non-sterile soil	19.1	29.6	0.4	23.4	72.5
Autoclaved soil	70.8	8.7	3.1	6.5	89.1
Fusarium solani	79.0	4.7	5.4	2.0	91.1
Fusarium episphaeria	75.6	2.1	2.0	1.3	81.0
Fusarium rigidiusculum	74.1	5.3	3.4	3.0	85.8
Penicillium lilacinum	74.4	6.7	5.2	4.8	91.1
Penicillium implicatum (green)	65.9	18.5	5.7	2.2	93.3
Penicillium implicatum (white)	79.2	10.8	2.0	2.0	94.0
Aspergillus elegans	73.4	7.4	3.4	1.6	85.8
SF-10	54.3[c]	5.2	16.1	14.6	90.2

[a] Carbaryl-^{14}C added to medium at 1 ppm in 1% ethanol.

[b] Percent of initial radioactivity (average of duplicate experiments).

[c] Percent of initial radioactivity (average of three experiments).

TABLE 36. RADIOACTIVITY OF MILES MEDIUM WITH CARBARYL-^{14}C[a] INOCULATED WITH BACTERIAL ISOLATES AND INCUBATED FOR 14 DAYS

	Radioactivity as			
Inoculum	Carbaryl	Water-solubles	Organo-solubles	Total
Medium control	66.1[b]	13.6	4.9	84.6
Bacillus sphaericus	79.1	13.9	6.7	99.7
SB-1	83.2	2.1	6.0	91.3
SB-2	84.9	4.5	5.4	94.8
SB-3	78.9	6.6	3.6	89.1

[a] Carbaryl-^{14}C added to medium at 1 ppm in 1% ethanol.

[b] Percent of initial radioactivity (average of 2 experiments).

TABLE 37. RADIOACTIVITY OF NUTRIENT BROTH WITH CARBARYL-^{14}C[a], INOCULATED WITH BACTERIAL ISOLATES AND INCUBATED FOR 14 DAYS

	Radioactivity as			
Inoculum	Carbaryl	Water-soluble	Organo-soluble	Total
Medium control	59.6[b]	24.4	3.9	87.9
Arthrobacter tumescens	53.5	33.1	5.7	92.3
Arthrobacter sp.	2.4	83.2	9.2	94.8
Nocardia flava	-	77.0	8.4	85.4
Pseudomonas acidovorans	1.0	76.9	1.1	79.0
Xanthomonas sp.	0.9	59.3	29.7	89.9
Bacillus sphaericus	9.1	27.1	41.7	77.9

[a] Carbaryl-^{14}C added to medium at 1 ppm in 1% ethanol.

[b] Percent of initial radioactivity (average of 2 experiments).

Carbofuran In Houseflies

The development of resistance to insecticides by insect pests has resulted in the use of greater quantities of toxic chemicals, the use of materials that are more toxic to man and, in some cases, a near complete inability to control certain pests. There is an ever-increasing need to more fully evaluate the whole phenomenon of insecticide resistance so that systematic approaches to developing control measures for these insects without increasing environmental and public health hazards may be formulated. Such evaluation can, and must, include a variety of investigational formats and emphases.

The insecticidal activity of carbamate insecticides is attributed largely to inhibition of the enzyme cholinsterase. When this enzyme is inhibited with a carbamate insecticide, the reaction is reversible and the enzyme can be reactivated. This happens in insects, and if recovery of the enzyme is too rapid, the insecticidal action of the carbamate is negated. This characteristic of carbamate inhibition of insect cholinesterase could play a part in the development of resistance to these chemicals by the pests. It is evident that if one population of insects could restore cholinesterase activity faster than another population, then their susceptibility to cholinesterase inhibitors would be less.

By demonstrating a difference in the metabolism and fate of an insecticide in susceptible and resistant populations, mechanisms responsible for these differences may be proposed and/or definitely defined. This then, would provide a basis on which to attack the problem of resistance. Should differences not be demonstrated, the need for greater attention to other areas such as enzyme-inhibitors stability would be apparent.

Methods and Materials

Carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranyl N-methylcarbamate-carbonyl-^{14}C) having a specific activity of 2 millicuries per millimole was applied topically to 6 day old houseflies at a dose of 0.04 micrograms per insect. The susceptible insects were from a laboratory strain of flies which previously had not been exposed to any insecticide. Resistant houseflies were originally taken from the susceptible strain but had been selected for resistance over a 2 year period by exposing each generation to increasing doses of the carbamate insecticide, Baygon. LD_{50} values for carbofuran against the susceptible and resistant populations were 0.1 and 1.3 micrograms per insect, respectively, at the time these experiments were initiated.

One-hundred each of the susceptible and resistant flies were treated with carbofuran-carbonyl-^{14}C for each of the time intervals selected for analysis. Each experiment was replicated 3 times. At the appropriate interval after treatment, the insects were anesthetized with carbon dioxide and the carbofuran remaining on the surface of the flies was removed by rinsing them with acetone. Internal radioactivity was extracted by homogenizing the flies in a 1:1 mixture of acetone and water. The radioactivity in the extract was partitioned into organo-soluble and water-soluble metabolites by the addition of chloroform. To determine the amount of carbofuran-^{14}C equivalents excreted by the insects, the holding vials were rinsed with acetone and the metabolites partitioned between chloroform and water.

The organo-soluble metabolites and the aglycones recovered from the water-soluble conjugates after acid hydrolysis were resolved by thin layer chromatography. Identification of the metabolites was based on comparative chromatographic analysis of the unknown materials and authentic samples of compounds considered as possible metabolites. The details of these techniques have been reported (Dorough 1968).

The heads of laboratory strains of insecticide-susceptible and carbamate-resistant flies served as the cholinesterase source for all studies unless otherwise stated. The resistant strain had been selected with the carbamate Baygon ^{R} for a 2-year period. LD_{50} values for Baygon against susceptible and resistant flies were 0.7 and 14.0 micrograms per insect, respectively.

Cholinesterase activity was determined by the colorimetric method of Ellman (1961). The inhibitory ability of carbaryl and Baygon was established by incubating the insecticides with the enzyme in phosphate buffer, for various periods at 30°C. The I_{50} values were determined after a steady state had been reached.

For a typical run, the insecticide, in acetone, was added to a 500-ml Erlenmeyer flask, and the acetone was removed by evaporation. Then 100 ml of the enzyme preparation, 0.03 fly-heads per ml of phosphate buffer, was added, and the incubation was initiated. At designated times, 3-ml samples were removed, and the level of cholinesterase inhibition was determined.

To determine the rate of reactivation of carbamate-inhibited cholinesterase, a concentrated enzyme solution, 48 fly-heads per ml, was incubated with sufficient insecticide to inhibit about 70-80 percent activity after 30 minutes. An aliquot was then removed and diluted 800-fold with the phosphate buffer, and the incubation was continued. This dilution prevented further enzyme inhibition by the insecticide and allowed the enzyme already inhibited to recover. The rate of recovery was determined by assaying 3-ml aliquots of the diluted solution at 10 min intervals once dilution had taken place.

Results and Discussion

It should be noted that the position of the carbon-14 atom in the carbofuran molecule prevented detection of ring-containing hydrolytic products. Upon hydrolysis, the carbon-14 atom would be released as carbon-14 dioxide in the respiratory gases. This may have been responsible for the decrease in the percentage of the dose recovered as the time after treatment increased. By 4 hours after treatment, total recovery was approximately 75% of the dose while it was in excess of 90% after only 1 hour. Other factors such as incomplete extraction of the residues from the flies also may have contributed to the loss.

The data demonstrated that carbofuran was absorbed faster by the susceptible flies. This was evident by the levels of carbofuran on the surface at each time of analysis and by the corresponding levels of carbofuran-carbonyl-^{14}C equivalents within the insects. At each interval, the surface wash of the resistant flies contained a greater percentage of the dose, while the internal ^{14}C-residues were always less than in the susceptible insects.

Of particular interest was the resistant insect's ability to excrete the absorbed carbofuran at a more rapid rate than the susceptible ones Even though more of the dose was absorbed by the susceptible flies, a greater portion of the dose was eliminated by the resistant flies. This enhanced ability to excrete any absorbed insecticide, combined with a slower absorption rate in the first place, would obviously result in less toxicant in the body of the resistance flies. Therefore a reduced toxic effect would be expected.

In this study, the two factors just mentioned were the only observations made which would explain, at least in part, the mechanism of carbofuran resistance in houseflies. The metabolism studies did not indicate that metabolic degradation was an important factor (Table 38). These data did, however, show that the reduced internal residues and increased excreted residues in the resistant flies were primarily a function of the parent compound, carbofuran, and not of its metabolites. The data suggest that the resistant flies can selectively excrete carbofuran more efficiently than the susceptible flies.

The concentration of either carbaryl or Baygon necessary to inhibit house fly-head cholinesterase was found to be the same in susceptible and carbamate-resistant house flies. Baygon was the more potent cholinesterase inhibitor as indicated by a $(I_{50})_{ss}$ value of 4×10^{-9}M; the (I_{50})ss value for carbaryl was 9×10^{-8}M. These data demonstrated that carbamate resistance was not related to a variation in the sensitivity of the enzyme to the insecticides.

The recovery rates of the susceptible and carbamate-resistant house fly cholinesterase after being inhibited at about the 80-percent level with Baygon and carbaryl were identical. Twenty-six minutes were required for 50 percent of the inhibited enzyme to be reactivated. Thus, our hypothesis that carbamate resistance could result, in part, from an enhanced ability of these insects to reactivate inhibited cholinesterase was not supported by the experimental data.

In order to prove that the techniques utilized were capable of showing differences in reactivation rates of carbamate-inhibited cholinesterases if such did occur, the enzyme from two other sources was subjected to the same tests as described above. Commercial bovine acetylcholinesterase inhibited with carbaryl and Baygon recovered at the same rate as the house fly-head cholinesterase. However, a 10-fold greater concentration of the insecticide was required to produce a $(I_{50})_{ss}$ with the bovine acetylcholinesterase. Cholinesterase from boll weevils show a different rate of reactivation than the enzyme from other sources. Once inhibited, 99 minutes was required before 50 percent of the enzyme was reactivated. Therefore, these experiments did show that differences could be detected if they occurred and also that rates of reactivation of carbamate-inhibited cholinesterase do vary in different insect species. The (I_{50})ss values for carbaryl and Baygon against boll weevil cholinesterase were essentially the same as for the house fly-head cholinesterase.

During the above investigations, a definite trend toward higher enzyme levels in the resistant flies was noted. Reviewing all assays, which included over 50 individual runs, it was found that the enzyme level of resistant flies was about 10 percent higher in 85 percent of the assays. As a result of these observations, experiments were conducted whereby the cholinesterase levels in the two strains of flies were compared directly.

The susceptible fly-heads hydrolyzed 8.6×10^3 micrograms of substrate, acetylcholine, per minute per gram of weight. Approximately 34 percent greater cholinesterase activity was obtained with the resistant fly-heads which hydrolyzed 11.5×10^3 micrograms of substrate per minute per gram. This degree of difference in enzyme activity was not apparent in earlier tests because the enzyme concentration was expressed on a fly-head per ml basis rather than on a weight basis. It was later found that the susceptible fly-heads weighed about 12 percent more than the resistant ones although the total body weight of flies from the two strains was the same.

TABLE 38. NATURE AND MAGNITUDE OF CARBOFURAN AND ITS METABOLITES IN THE BODY AND EXCRETA OF SUSCEPTIBLE (S) AND CARBAMATE RESISTANT (R) HOUSEFLIES FOLLOWING TOPICAL APPLICATION OF THE INSECTICIDE

	Percent of applied dose at indicated hours							
	Body				Excreted			
	1		4		1		4	
Metabolites	S	R	S	R	S	R	S	R
Organo-extractables								
Carbofuran	9.3	5.5	6.4	4.4	5.3	8.3	6.2	12.7
3-OH-Carbofuran	5.4	1.9	1.9	1.3	0.1	0.2	1.6	1.0
3-OH-NCH$_2$OH-Carbofuran	0.9	0.7	0.3	0.4	0	0	0.5	0.9
3-Keto-Carbofuran	4.3	2.3	0.9	0.5	0	0	0	0
Unknown I[a]	0.2	0.1	0.2	0.1	0.1	0.1	0.1	0.1
Total	20.1	10.5	9.7	6.7	5.5	8.7	8.4	14.7
Water-solubles								
3-OH-Carbofuran	10.0	7.9	10.4	6.5	1.1	2.0	0.2	0.2
3-OH-NCH$_2$OH-Carbofuran	1.3	0.3	0.8	0.4	0	0.1	0.5	0.6
NCH$_2$OH-Carbofuran	0.9	0.9	1.5	1.2	0	0.4	1.5	1.9
3-keto-Carbofuran	0.8	0.3	0.4	0.3	0	0	0.2	0.2
Unknown I	0.4	0.4	1.0	0.4	0.1	0.2	1.3	1.0
Unknown II[b]	1.7	1.9	4.2	2.6	0.2	0.6	4.6	4.2
Total	16.0	11.7	18.3	11.4	1.4	3.3	8.3	8.1

[a] Radioactivity remaining at the origin after development of the tlc.

[b] Metabolites in the aqueous layer following acid hydrolysis and extraction with chloroform.

FATE OF CARBAMATE METABOLITES

1-Naphthyl Glucoside in Rats

Based upon persistence and potential harmful effects from chronic exposure, the conjugate metabolites of the carbamate insecticides may prove to be quite significant in animals. To date, little information exists on the fate of these conjugates in various species and nothing is known of their chronic toxicity. One of the principal reasons that so little information is available is that the compound requires unique methods of isolation, identification and synthesis. Unkike the apolar insecticide metabolites, the conjugated materials can not be extracted into an organic solvent and subjected to conventional tlc, glc, etc.

This study was conducted to develop techniques basic to the study of the chemistry and metabolism of conjugate metabolites. 1-Naphthol was selected as a model compound, although it is also a metabolite of the insecticide carbaryl.

Methods

Chemical synthesis of 1-naphthyl glucoside - The glucoside of 1-naphthol was prepared by reacting glucose pentaacetate with 1-naphthol to form 1-naphthyl-tetra-O-acetyl-β-D-glucopyranoside. Details of the synthesis procedures were as follows:

Glucose pentaacetate (3.9 g, 0.01 mol) was mixed with 4.3 g (0.03 mol) of 1-naphthol and 0.2 g of p-toluenesulfonic acid. The mixture was heated, under vacuum, for 1 hr in an oil bath at 100°C. Following cooling to room temperature, the melt was dissolved in 100 ml of benzene and extracted twice with 20 ml-portions of 2% sodium hydroxide. The benzene was dried with anhydrous sodium sulfate and then concentrated to dryness. Crystallization from ethanol and water gave 4.5 g of the 1-naphthyl-tetra-O-acetyl-β-D-glucopyranoside, mp 177-178° (Lit. 178-179°). Deacetylation was accomplished by passing dry ammonia gas through a methanol solution of the product (2.0 g/10 ml) for 1 hr at 0° and then holding the solution at 5° for an additional 3 hrs. The methanol was evaporated and the residue crystallized from a mixture of ether and ethanol. 1-Naphthyl-β-D-glucopyranoside, 1.2 g, was recovered as a white powder, mp 171-175°. The mass spectrum (Finnigan Model 1015C) contained a weak molecular ion at m/e 306 while the base peak occurred at m/e 144 due to 1-naphthol. Peaks at m/e 116 and 115 represented the base peak minus CO and CHO, respectively. The sugar moiety was evidenced by peaks at m/e 163 ($C_6H_{11}O_5$ +) and 162 ($C_6H_{10}O_5$ +).

Biosynthesis of radioactive 1-naphthyl glucoside and glucuronide - For the synthesis of 1-naphthyl-1-^{14}C-β-D-glucopyranoside, an in vitro glucosylation system using house fly enzymes was employed (Mehendale and Dorough, 1972). Each incubation mixture consisted of 0.5 uCi of 1-naphthol-1-^{14}C (specific activity 19.6 mCi/mmol), 2.4 ml of Tris-HCl buffer (pH 7.2), 1 ml of 0.1 M magnesium chloride, 0.5 ml of 5 mg/ml solution of UDPG dissolved in buffer, and 1 ml (100 mg tissue equivalents) of a 9,000 g supernatant of a house fly homogenate. Incubations were for 15 min at 37°C.

The incubation mixture was extracted thoroughly with ether to remove the unreacted 1-naphthol and the water layer was concentrated to a volume suitable for application to thin layer chromatograms. After development, the 1-naphthyl-1-^{14}C glucoside was detected on the chromatograms by radioautography and the compound recovered by extraction of the gel with methanol. Approximately 75% of the radioactive 1-naphthol was converted to the glycoside.

1-Naphthyl-β-D-glucopyranoside-^{14}C also was synthesized enzymatically using the basic procedure just described. However, an excess of 1-naphthol, 1 mg/flask, was used to achieve maximum reaction with the

1 uCi of uridine diphosphate glucose [D-glucose-^{14}C (U), sp. act. 227 mCi/mmole] added to each incubation mixture. Under these conditions, 35% of the radiocarbon was recovered as 1-naphthyl glucoside-^{14}C.

Radioactive 1-naphthyl glucuronide was prepared enzymatically using rat liver homogenates as the enzyme source and UDPGA as the co-factor (Mehendale and Dorough, 1971). The 1-naphthol-^{14}C used in these experiments was the same as that used to prepare the glucoside. 1-Naphthyl glucuronide-^{14}C was synthesized from uridine diphosphate glucuronic acid [D-glucuronic-^{14}C (U)] having a specific activity of 238 mCi/mmol.

Acetylation of biosynthesized 1-naphthyl glucoside - 1-Naphthyl-^{14}C glucoside was synthesized using house fly enzymes as described earlier and the unreacted 1-naphthol-^{14}C removed by extracting the incubation mixture with ether. The water phase, containing the radioactive naphthyl glucoside, was concentrated to dryness on a rotary evaporator. The dried residue was dissolved in 1 ml of acetic anhydride and cooled to 0°C in an ice bath. Two drops of 70% perchloric acid were added and the solution allowed to return to room temperature slowly over a period of 2 hr. At this stage, the solution was held overnight and then transferred to a separatory funnel containing 4 ml of cold water. The water was extracted twice with 4 ml-portions of ether which removed 85% of the radioactivity, presumably the acetylated naphthyl glucoside. Its identity was confirmed by cochromatography on tlc with the chemically synthesized material.

Deacetylation of the product with ammonia converted 93% of the radioactivity to a water soluble material. Tlc analysis demonstrated that this material was identical to an authentic sample of naphthyl glucoside.

Chromatography - 1-Naphthol was purified on silica gel F-254 chromatoplates (0.25 mm, EM Lab, Elmsford, N. Y.) using a 7:3 mixture of chloroform and acetone as the developing solvent. In addition to

Providing a means of purification, this system afforded the separation of 1-naphthol; 1,3-naphthalenediol; 1,4-naphthalenediol; 1,5-naphthalenediol and 1,6-naphthalenediol. These compounds constituted the more likely free metabolites of 1-naphthol resulting from treating rats with 1-naphthyl glucoside. Other solvent systems used to establish co-chromatography of an unknown with one of these metabolite standards were: petroleum either-ethyl ether 2:1; methylene chloride-ethyl acetate 2:1; hexane-acetone 7:3.

Chromatographic isolation of acetylated naphthyl glucoside (1-naphthyl-tetra-O-acetyl-beta-D-glucopyranoside) was accomplished on silica gel chromatoplates developed in 10:1 petroleum ether-acetone. The acetylated naphthyl glucoside had an R_f of approximately 0.6 while the naphthyl glucoside remained at the origin. Changing the solvent to 8:2:1 chloroform, methanol and acetic acid moved the acetylated naphthyl glucoside to the solvent front but resulted in an R_f of 0.6 for the naphthyl glucoside. Naphthol glucuronide had an R_f of 0.4 in this system while UDPG and UDPGA remained at the origin. This allowed the separation of the ^{14}C-labeled biosynthesized glycosides from their corresponding radioactive cofactors.

Non radioactive 1-naphthol and its hydroxylated analogs were detected on the tlc plates by viewing under ultraviolet light. To locate the glycosides, the chromatograms were sprayed with concentrated sulfuric acid and heated at 110°C for 5 min. Radioautography (Kodak no-screen medicat x-ray film) was used to detect radioactive areas on the gel. Quantitative radioassay were conducted by liquid scintillation counting.

Stability of 1-naphthyl glucoside and glucuronide - The stabilities of 1-naphthyl-^{14}C glucoside and 1-naphthyl-^{14}C glucuronide were evaluated under various conditions commonly used for evaluating the chemical and/or biological fate of insecticidal compounds. Each of the radioactive glycosides were purified on tlc prior to use in these experiments. The situations under which the glycosides were held for stability determination were: (1) in citrate-phosphate buffer, pH ranging from 2.2 to 8.0, for up to 6 hr at room temperature. (2) In Tris-HCl buffer, pH 7.0, at temperatures of 25, 0 and -20°C for up to 1 week and at 90°C for 30 min. (3) In methanol at 0.25 and 65°C for 12 hrs and at -20°C for 5 days. (4) In methanol and refluxed for 30 min. At the designated time, the incubation mixtures were concentrated for tlc analysis.

Enzyme studies - The ability of β-glucosidase and β-glucuronidase (Sigma Chemical Corp. St. Louis, Mo.) to hydrolyze naphthyl glucoside and glucuronide was determined. The naphthyl-^{14}C glycosides were incubated separately with each of the enzymes in citrate-phosphate buffer, pH 7.0. The incubation mixtures containing approximately 1.0×10^6 dpm of the glycoside and 4 ml of buffer, were preincubated, with shaking, for 10 min at 37°C and then 0.4 mg of enzyme in 0.2 ml of buffer were added. The same amount of enzyme was added 4 more times at 6 min intervals with the total incubation time being 30 min. Each reaction mixture was extracted twice with 5 ml portions of ether and both the aqeuous and organic solvent phases were radioassayed. The quantity of radiocarbon in the ether was indicative of the efficiency of the enzyme to cleave the glycoside, thus yielding radioactive 1-naphthol.

To determine if whole rat urine affected the enzymatic cleavage of naphthyl glucoside or naphthyl glucuronide, the reactions described above were run in the presence of 0.5 ml of urine. In this case, however, the initial buffer volume was increased to 5 ml and the pH adjusted to 7.0 if required after adding the urine. In addition, each of the glycosides were treated in succession with the 2 enzymes, first with glucosidase and then with glucuronidase and vice versa. This was done to determine if the sequential treatment of a single urine sample with the enzyme would produce results comparable to treatment of the sample with either of the enzymes alone.

Fate of 1-naphthyl glucoside in rats - Female rats weighing approximately 250 g were treated orally with 1-naphthol-^{14}C in corn oil or with water solutions of carbon-14 1-naphthyl glucoside. For the latter studies, the 1-naphthyl glucoside was labeled in 2 different positions. One batch was labeled on the naphthyl ring while the other batch was labeled on the sugar moiety. The animals were held in metabolism cages and the urine collected for 24 hr and the nature of the radiocarbon determined.

Results and Discussion

Stability of 1-naphthyl glucoside and glucuronide - Both naphthyl-^{14}C glucoside and glucuronide were stable when held in citrate-phosphate buffer at pH values ranging from 2.2 to 8.0 for 6 hr and at a pH of 7.0 when heated in a water bath at 90°C for 30 min. The same was true when the compounds were dissolved in methanol and stored at -20, 0 and 25°C for 5 days, or when the solutions were refluxed for 30 min. Storage at -20, 0 and 25°C in Tris-HCl buffer for 1 week did not degrade the naphthyl glucoside. The glucuronide derivatives were similarly stable at the two lower temperatures but 30% degradation did occur at 25°C. At the latter temperature, the radiocarbon not associated with glucuronide was identified by tlc analysis as 1-naphthol.

The data indicate that no particular problems would be encountered insofar as stability is concerned during extraction of the compounds from biological media. Their stability in methanol is important since this is an excellent solvent for extracting polar insecticide metabolites from plant and animal tissues. Enzyme studies, such as the cleavage of the conjugates to form the aglycones, must be conducted using the appropriate control if specificity of the enzyme is to be considered. Since some cleavage of the naphthyl glucuronide did occur when held in

Tris-HCl buffer, pH 7.0, at 25°C, non-enzymatic cleavage must be considered a possibility at incubation temperatures above room temperature, especially if the incubation time is of long duration. The citrate-phosphate buffer may be the preferred buffer in such incubation since both glycosides were stable when held in this buffer for 6 hr at 25°C.

Enzyme studies - Incubation of 1-naphthyl-^{14}C glucoside with β-glucosidase for 30 min at 37°C cleaved 91% of the conjugate. β-Glucuronidase treatment yielded only 3% cleavage which was comparable to a control incubation containing all constituents except the enzyme. 1-Naphthyl glucuronide was hydrolyzed in excess of 95% by β-glucuronidase and 7% by β-glucosidase. The latter enzyme did not cleave the conjugate to any greater degree than that observed during incubation with no enzyme.

Whole rat urine, 0.5 ml, added to the enzyme preparation did not decrease the amount of substrate hydrolyzed, nor did it alter the specificity of the enzymes. Also, β-glucuronidase in the incubation mixture did not affect the acti[illegible] of β-glucosidase on 1-naphthyl glucoside. The same was true when the situation was reversed.

From this study, it appears that β-glucosidase and β-glucuronidase treatment of a biological extract could be used as a convenient tool for determining the ability of an organism to metabolize a glucoside to a glucuronide. For example, a rat fed 1-naphthyl glucoside might be expected to convert the material to a glucuronide and excrete the compound in the urine. Treatment of the urine with β-glucosidase, followed by β-glucuronidase, would allow one to rapidly establish the quantity of the dose eliminated or the administered compound and the quantity metabolized to form the glucuronide. This would be an immediate indication of the animal's ability to attack, biochemically, plant-derived glucosides of the chemical in question if consumed in the diet. Identification of the resulting aglycones would give virtual proof of the intact conjugates as they exist in the plant and animal system.

Fate in rats - When 1-naphthyl-^{14}C glucoside was administered as a single oral dose to rats, 67% of the dose was eliminated in the urine after 24 hrs (Table 39). Approximately 90% of a dose of 1-naphthol-^{14}C was eliminated during the same period.

A rather surprising aspect of the study with 1-naphthyl-^{14}C glucoside was that 19% of the dose was eliminated as the administered compound. Thus, this glucoside metabolite did withstand the acid conditions of the stomach and the biochemical mechanism of degradation in the body. That the sugar moiety was the same as administered (as opposed to cleavage of the conjugate to yield 1-naphthol, followed by re-conjugation as a glucoside in the animal) was confirmed in 2 ways. First, 1-naphthol-^{14}C treatment yielded only trace amounts of radioactivity in the urine which corresponded to 1-naphthyl glucoside. Secondly, 16% of a dose of 1-naphthyl glucoside-^{14}C was in the 0-24 hr urine as the administered compound. These data clearly showed that the rat does not form glucosides, at least to any appreciable extent, and that the 1-naphthyl glucoside in the urine represented the administered compound.

Cleavage of the 1-naphthyl glucoside in the rat to yield 1-naphthol was a major metabolic pathway. About 10% of the dose was in the urine as 1-naphthol, 24% as 1-naphthyl glucuronide and 10% as 1-naphthyl sulfate. The relative concentrations of these metabolites were quite different than in the urine of the 1-naphthol-^{14}C treated rats. Only 1% of the dose was as the free 1-naphthol, 73% as the glucuronide and 15% as the sulfate.

Treatment of rats with 1-naphthyl glucoside-^{14}C confirmed that the 1-naphthyl glucuronide in the urine of rats administered the glucoside did not result from the oxidation of the glucoside. Only a small amount, 1% of the dose, of radioactivity corresponded to 1-naphthyl glucuronide, a level so small that confirmation of its identity was not possible. This demonstrates that the glucoside was first cleaved to yield 1-naphthol, which was then conjugated as a glucuronide in the animal system.

This study with 1-naphthyl glucoside does not represent a metabolite of high potential for significance. However, the study has allowed us to develop techniques that will assist us in studying more potentially significant metabolites, such as glucosides of metabolites of carbaryl containing the carbamate moiety. More important, the study did establish that there is a definite need to evaluate the significance of the conjugates, per se, rather than relying entirely on information obtained with the corresponding aglycone.

TABLE 39. NATURE OF RESIDUES IN THE 0-24 HOUR URINE OF RATS TREATED ORALLY WITH 1-NAPHTHOL AND 1-NAPHTHYL GLUCOSIDE

	% of dose when treated with		
		1-Naphthyl glucoside	
Metabolites	1-Naphthol-1-^{14}C	Naphthyl-^{14}C	Glucoside-^{14}C
1-Naphthyl Glucoside	0.1	18.7	15.9
1-Naphthyl Glucuronide	73.2	23.6	1.1
1-Naphthyl Sulfate	14.9	10.3	0
1-Naphthol	1.4	9.5	0
Unknown (tlc origin)	0.7	5.2	3.1
TOTAL	90.3	67.3	20.1

3-Hydroxy Carbofuran and its Glucuronide and Glucoside in Rats

Many investigators have demonstrated that 3-hydroxy carbofuran is a major metabolite of carbofuran in most biological systems. In mammals, the 3-hydroxy carbofuran is further metabolized by conjugation to the corresponding glucuronide. In plants, a glucoside is formed. These 3 metabolites constitute a large proportion of the terminal residues in the environment resulting from the introduction of the pesticide carbofuran. Because the 3-hydroxy analog of carbofuran is highly biologically active, it is important to know the fate of this metabolite in animals and plants.

Methods and Materials

Chemicals - Carbofuran, 3-hydroxy carbofuran and carbofuran-carbonyl-^{14}C (17 uCi/mg) were supplied by Niagara Chemical Division, FMC Corp., Middleport, N. Y. Each of the chemicals was chemically pure as evidenced by the existence of only one spot on thin layer chromatography. TPNH and UDPG were purchased from Sigma Chemical Company, St. Louis, Missouri.

Metabolism by rat liver enzymes - A 50% rat liver homogenate (w/v) in 0.05 M Tris-HCl buffer (pH 7.0) was prepared and separated into two fractions. One fraction was prepared by centrifuging the homogenate at 15,000g for 30 minutes; the supernatant was used as the enzyme source for the oxidative metabolism of carbofuran. The other fraction was prepared by centrifuging the 15,000g supernatant at 105,000g for 1 hour. The particulate fraction (microsomes) was used as the enzyme source for conjugation of 3-hydroxy carbofuran after dispersal in a volume of Tris-HCl buffer that made the suspension equivalent to the original 50% homogenate.

The 3-hydroxy carbofuran-carbonyl-^{14}C was produced from the oxidation in vitro of carbofuran-carbonyl-^{14}C. In a typical incubation, 1 ml of the enzyme source and 2 ml of the pH 7.4 Tris-HCl buffer containing 4 umoles of TPNH were added to a 25 ml Erlenmeyer flask containing carbofuran-carbonyl-^{14}C. After incubation at 37°C in a water bath shaken for 30 minutes, the contents of each flask were extracted three times with 5 ml portions of ether, and the ether extracts were combined and dried with anhydrous sodium sulfate. Aliquots from both the water and organic solvent phases were radioassayed using liquid scintillation technique. Ether extracts were evaporated to about 0.2 ml and spotted on tlc plates (0.25 mm thick). The plates were developed in a 3 to 1 ether-hexane mixture, exposed to x-ray film for 3 days, and the areas of the gel corresponding to darkened areas on the radioautogram were extracted into ether from the gel.

To determine the maximum quantity of carbofuran metabolites produced, the 15,000g soluble fraction was incubated with concentrations of carbofuran ranging from 20 to 1000 ug. The 3-hydroxy carbofuran-carbonyl-^{14}C was isolated from the tlc and identified by cochromatography.

The glucuronide and glucoside conjugation of 3-hydroxy carbofuran were investigated using the liver microsomes and housefly homogenates. For glucuronide conjugation, the incubation mixture contained 3-hydroxy carbofuran-carbonyl-^{14}C, $MgCl_2$ (100 umoles, in 1 ml Tris-HCl buffer of pH 7.0); UDPGA (2 umoles, in 1 ml Tris-HCl buffer of pH 7.0) and liver microsomes (equivalent to 250 mg liver). The incubation was carried out aerobically at 37°C for 20 minutes. Free metabolites were extracted into ethyl ether and the percent radioactivity remained in the water phase served as a measure of the formation of 3-OH-carbofuran-carbonyl-^{14}C glucuronide. To investigate the effects of substrate level and incubation period on conjugation, various concentrations of 3-OH carbofuran (0.1 to 1.1 umole) as well as three different incubation times were employed. The same parameters and method was used for glucoside conjugation of 3-OH carbofuran-carbonyl-^{14}C except that a housefly homogenate (15,000g soluble, equivalent to 250 mg wet weight) and UDPG (50 mg of 1 ml tris-HCl buffer, pH 7.0) were used in place of the liver microsomes and UDPGA.

To determine if the rat liver enzymes could alter carbofuran glucuronide, the material was incubated in the presence of the enzyme source, TPNH and UDPGA. After incubation, the organosoluble materials formed, if any, were extracted into ethyl ether. The ether soluble radioactivity was condensed to 0.2 ml and applied to a silica gel G tlc and developed in a 3 to 1 ether-hexane mixture. The water soluble radioactivity was applied to a silica gel G tlc and developed in 3:1 chloroform-methanol containing 10% acetic acid.

Fate in rats - Female Sprague-Dawley rats, weighing approximately 200 gm, were given oral doses of 3-hydroxy carbofuran-carbonyl-^{14}C in corn oil or 3-OH carbofuran-carbonyl-^{14}C glucuronide or glucoside in water. The urine, feces and $^{14}CO_2$ were collected for up to 144 hours after the single doses. The feces were dried at 50°C and ground and a 100-mg aliquot collected at each period was combusted in a Beckman Biological Material Oxidizer; the $^{14}CO_2$ produced was trapped in a solution of methyl cellusolve and 2-aminoethanol (2:1). The $^{14}CO_2$ produced from respiration was collected from the animals using the same trap solution.

The metabolites in the urine collected within 32 hours of treatment were also analyzed. The urine was extracted 4 times with a 3 to 1 mixture of chloroform-acetonitrile. The organosoluble extracts and the water layer were concentrated and analyzed by tlc as described earlier.

Results and Discussion

The optimal concentration of carbofuran-carbonyl-^{14}C required for the formation of 3-hydroxy-carbofuran-carbonyl-^{14}C by the rat liver homogenate was 50 to 100 micrograms (Table 40).

The formation of glucuronide and glucoside of 3-hydroxy carbofuran-carbonyl-^{14}C by the liver microsome preparation (105,000 x g) and housefly homogenate (15,000 x g) did not exceed about 20% regardless of the parameters (Tables 41, 42).

When the enzymatically synthesized glucuronide conjugate of 3-hydroxy carbofuran was subjected to enzyme hydrolysis with β-glucuronidase at 37°C, 85% of the radioactivity was recovered as 3-hydroxy carbofuran.

Metabolism of 3-hydroxy-carbofuran-carbonyl-^{14}C and glycosides in rats - It is generally thought that a compound with a polar group or groups is metabolized and excreted faster from animals than less polar compounds. The results of this investigation werein agreement with the principle. When the glucuronide of 3-hydroxy-carbofuran was administered orally to rats, 80% of the dose was excreted in the urine within 48 hours, 16% in the feces and 4% as $^{14}CO_2$. With 3-hydroxy carbofuran-carbonyl-^{14}C, 34% of the dose was in the urine, 28% in the feces and 4% as $^{14}CO_2$ by the end of 48 hours after the treatment. The glucuronide conjugate of 3-hydroxy-carbofuran was also excreted faster than its aglycone. After 48 hrs, the urine contained 42% of the dose, the feces 18% and about 4% was as $^{14}CO_2$.

The organo-extractable and the water soluble metabolites from the urines collected 32 hours after treatment were analyzed using thin layer chromatography. Water soluble metabolites were the most portion of the radioactivity in the urine (Table 43). The percentage as water solubles was 61% in urine from rats receiving 3-hydroxy-carbofuran and 82% in the urine of rats receiving the glucuronide and 91% in the urine of rats treated with the glucoside. In the chloroform extract, only one radiolabeled metabolite was detected and this was identified as 3-hydroxy-carbofuran.

Metabolism of 3-hydroxy-carbofuran-carbonyl-^{14}C in bean plants - Bean leaves immersed in an acetone-water (1:20) solution containing 3-hydroxy-carbofuran-carbonyl-^{14}C for 6 days were extracted and partitioned as described for the rat urine. Thirty-five percent of the radioactivity in the leaves were extracted into the chloroform-acetonitrile and consisted of 3-hydroxy-carbofuran. Of the 65% radiocarbon in the water, only one metabolite was detected on the thin layer chromatograms. This spot occupied a higher position on the thin layer chromatogram than the glucuronide of 3-hydroxy-carbofuran and was tentatively identified as 3-hydroxy carbofuran-carbonyl-^{14}C glucoside. Upon enzymatic hydrolysis with glucosidase, 3-hydroxy-carbofuran-carbonyl-^{14}C was recovered in the ether extract.

TABLE 40. INFLUENCE OF VARIOUS LEVELS OF CARBOFURAN ON THE METABOLISM OF CARBOFURAN-CARBONYL-^{14}C BY THE 15,000g SOLUBLE FRACTION OF RAT LIVER

Micrograms of carbofuran	Percent of added radioactivity as metabolites			
	3-OH carbofuran	3-keto carbofuran	carbofuran	Water solubles
20	41.3	1.6	37.2	19.8
50	42.8	2.1	36.0	16.1
70	42.8	2.1	41.0	14.1
100	43.0	2.8	42.4	11.2
200	35.2	2.5	51.2	11.1
300	38.2	1.4	40.6	13.6
500	37.4	1.5	46.3	10.8
700	28.9	1.0	59.6	10.4
1,000	27.8	2.1	61.8	8.1

TABLE 41. EFFECT OF SUBSTRATE CONCENTRATIONS ON GLUCURONIDATION OF 3-HYDROXY-CARBOFURAN-CARBONYL-^{14}C BY THE 105,000g FRACTION OF RAT LIVER

Micrograms 3-OH carbofuran	Percent glucuronidation
Background[a]	4.6
23	20.7
70	18.0
117	16.3
165	14.7
210	14.4
260	14.3

[a] Without microsomes.

TABLE 42. EFFECT OF VARIOUS INCUBATION PERIODS ON THE GLUCURONIDE AND GLUCOSIDE CONJUGATION OF 3-HYDROXY-CARBOFURAN-CARBONYL-^{14}C BY A RAT LIVER (15,000g SOLUBLE) AND A HOUSEFLY HOMOGENATE (15,000g SOLUBLE)

Incubation period, min.	% 3-OH-carbofuran converted to Glucuronide	% 3-OH-carbofuran converted to Glucoside
20	21.5	6.8
40	18.8	19.4
60	18.9	14.3

TABLE 43. PARTITIONING CHARACTERISTICS OF METABOLITES IN THE 0-32 HOUR URINE OF RATS TREATED ORALLY WITH 3-OH-CARBOFURAN OR ITS GLUCURONIDE OR GLUCOSIDE DERIVATIVES[a]

	Percent of radioactivity in sample as	
Treatment	Organosolubles	Watersolubles
3-OH-carbofuran	38.6	61.4
3-OH-carbofuran glucuronide	18.2	81.8
3-OH-carbofuran glucoside	9.5	90.5

[a] Approximately 60,000 dpm of each material administered to the rats. The glucuronide was biosynthesized using rat liver microsomes and the glucoside isolated from 3-OH-carbofuran-treated bean plants.

INTERACTIONS

Effect of Aldicarb on Methyl Parathion Toxicity to Mice

Aldicarb, a carbamate insecticide, showed only additive toxic effects to mice when administered along with methyl parathion, an organophosphorus insecticide. The oxime hydrolytic product of aldicarb, which is nontoxic, did not alter the toxicity of methyl parathion in any way, nor was the protective action of the antidotes, atropine and 2-PAM, influenced by the addition of the oxime derivative of aldicarb.

Atropine and 2-PAM (2-pyridine aldoxime methiodide) are the two antidotes commonly employed in the treatment of poisoning by anti-cholinesterase agents such as methyl parathion (Hayes, 1963). 2-PAM is one of the several oximes which have the unique capability of promoting the release of enzymes bound by the toxicants. Hayes reports that in most cases of parathion poisoning in man a single dose of 2-PAM was sufficent to produce dramatic improvement within 30 minutes.

Aldicarb [2-methyl-2-(methylthio)propionaldehyde O-(methylcarbamoyl) oxime] is a carbamate insecticide and a very potent cholinesterase inhibitor. As the chemical name shows, it also is an oxime derivative, making it somewhat similar in chemical configuration to the antidote. 2-PAM. While aldicarb obviously does not act as an antidote against itself, the possibility exists that it could antagonize the toxic effect of other cholinesterase inhibitors. The idea is made even more intriguing by the fact that the hydrolysis of aldicarb yields aldicarb oxime [2-methyl-2-(methylthio) propionaldehyde-oxime]. This is a non-toxic oxime derivative and is formed from aldicarb in mammalian systems (Andrawes et al., 1967). The similarity of aldicarb, and especially aldicarb oxime, to the chemical configuration of 2-PAM provided the stimulus for the study reported herein.

Methods

Female Yale Swiss mice approximately 4 weeks old and weighing from 20 to 25 grams were used in all tests. The animals were housed under continuous light at a temperature of 78°F prior to and during the toxicity determinations.

For both oral treatment and interperitoneal, I.P., injections of the compounds into the mice, a total volume of 0.1 ml was administered. Refined cotton seed oil served as the carrier for methyl parathion, aldicarb and aldicarb oxime. Atropine sulfate and 2-PAM were dissolved in water for administration. In all experiments, the mice were treated first with methyl parathion and then 20 minutes later with the other compound(s). The percentage mortality of the mice was recorded 24 hours after treatment. Ten animals were treated at each dosage level, and all experiments were replicated 3 times.

Results and Discussion

In order to obtain a baseline from which to work, it was necessary to determine the toxicity of all compounds used in the study to the mice when administered alone. It was found that aldicarb oxime and 2-PAM given at a dose of 25 mg/kg and atropine at a dose of 50 mg/kg did not cause any ill effects to the mice. This was true for both oral and I.P. treatments.

Methyl parathion and aldicarb were toxic to the mice and the dose of each required to kill 50% of the animals, LD_{50}, was determined to be as follows:

	LD50	
	Oral	I.P.
Methyl parathion	40 mg/kg	10 mg/kg
Aldicarb	1.5 mg/kg	0.3 mg/kg

These data were used as the basis for expressing the effects of the various compounds on the toxicity of methyl parathion to the mice. For example, the data in Table 44 shows that the effect of aldicarb on the toxicity of methyl parathion was "additive". This means that treatments of the mice with one-half the LD_{50} dose of methyl parathion and one-half the LD_{50} dose of aldicarb gave approximately 50% mortality. If less than 50% mortality occurred, the situation was referred to as "antagonistic" and if more than 50 percent mortality occurred, it was referred to as "synergistic" action. The latter case was not observed with any of the combinations used in these studies. An effect listed as "none" in Table 44 means that the observed toxicity to the mice was at the level expected from one-half the LD_{50} dose of methyl parathion.

Whereas the carbamate insecticide, aldicarb, had an additive effect on the toxicity of methyl parathion, aldicarb oxime did not in any way influence the toxicity of the organophosphorus material. It was evident at this point that neither aldicarb nor its oxime possessed the antidotal properties of the oxime, 2-PAM. There remained, however, the possibility that aldicarb oxime might enhance the antidotal action of 2-PAM and atropine.

When methyl parathion and 2-PAM were administered by I.P. injection, there was an 8-fold antagonistic action observed. In other words , 8 times the LD_{50} dose of methyl parathion was required to kill 50% of the animals pre-treated with 2-PAM. There was only a slight antagonistic effect, less than 2 fold, when methyl parathion was administered orally and 2-PAM administered either orally or by I.P. injection. This same low level of protection was provided by 2-PAM containing aldicarb oxime, suggesting that aldicarb oxime did not act to enhance the action of 2-PAM. Atropine alone gave only a 5-fold antagonistic action against the toxic effects of methyl parathion. Aldicarb oxime did not increase its capability as an antidote. Results of a test not included in Table 44 showed that 2-PAM did not affect the toxicity of aldicarb to the mice.

The most efficient antagonist of methyl parathion toxicity to mice was a mixture of atropine and 2-PAM. From 80 to 100 times the LD_{50} dose of methyl parathion was required to kill 50% of the mice receiving pretreatments of this mixture. It was clear that atropine and 2-PAM did interact to provide greater protection to the mice than when either was given alone.

TABLE 44. EFFECT OF ALDICARB, ALDICARB OXIME, 2-PAM, ATROPINE AND CERTAIN MIXTURES OF THESE COMPOUNDS ON THE ACUTE TOXICITY OF METHYL PARATHION TO MICE

Methyl Parathion (A) Plus (B)	Dose of B, mg/kg	Route of administration	Effect of B on toxicity of A
Aldicarb[a]	1/2 LD_{50}	A, B - Oral	Additive
Aldicarb oxime	25	A, B - Oral	None
Aldicarb oxime	25	A - Oral, B-I.P.	None
2-PAM	25	A, B - Oral	Antagonistic (< 2-fold)
2-PAM	25	A, B-I.P.	Antagonistic (8-fold)
2-PAM	25	A - Oral, B-I.P.	Antagonistic (< 2-fold)
2-PAM + Aldicarb oxime	25 + 25	A - Oral, B-I.P.	Antagonistic (< 2-fold)
Atropine	50	A - Oral, B-I.P.	Antagonistic (5-fold)
Atropine + 2-PAM	50 + 50	A, B-I.P.	Antagonistic (100-fold)
Atropine + 2-PAM	50 + 50	A - Oral, B-I.P.	Antagonistic (5-fold)

[a] Methyl parathion and aldicarb administered at one-half their LD_{50} doses.

Modification of Carbaryl Metabolism with Monoamine Oxidase Inhibitors

Selective control of the metabolic fate of insecticides in mammalian and insect systems provides one of the most promising methods of improving and maintaining the efficacy and safety of these chemical toxicants. By inhibiting metabolic detoxication mechanisms in the pest species or enhancing the mechanisms in the nontarget species through use of other chemicals, an ineffective compound can become an effective one, a nonspecific toxicant can become more selective, and the level of toxicant required to control the pest species may be lessened considerably. Currently, many compounds are referred to as insecticidal synergists and have been used for years with limited success to accomplish these desirable features.

Insecticidal synergists are thought to enhance the toxic action of insecticides as a result of their ability to inhibit the mixed-function oxidase system of microsomes. This explanation for the mode of action is a rather recent development and followed the successful use of insecticidal synergists by many years. The action of insecticidal synergists and a thorough discussion of their history and potential were reported by Casida (1970). In insecticidal synergists in use today, the active compounds were discovered first and their action described later. Obviously, if certain compounds can produce desirable effects by inhibiting a specific type of metabolic reaction, namely oxidation, probably other compounds exist which would produce a similar effect by inhibiting any important metabolic process. Other processes which are of importance in the metabolism of foreign compounds include hydrolysis, reduction, and conjugation (Williams 1963).

Because conjugation is so important in the metabolism of the carbamate insecticides (Dorough, 1970; Kuhr, 1970), this mechanism of metabolism was selected for inclusion in studies designed to determine the effect of certain chemicals on metabolic processes. Monoamine oxidase inhibitors were chosen because of their known interactions with other compounds (Brodie et al. 1958; Stockley 1969 a,b) and, more specifically, because of their reported ability to inhibit the conjugation of o-aminophenol by rat liver slices and rabbit liver homogenates (Hargreaves, 1968). Seven of these drugs were shown recently to be inhibitors of carbaryl metabolism by a 15,000g soluble fraction of rat liver (Culver et al., 1970). The present study was undertaken to determine in vivo effects of several monoamine oxidase inhibitors on carbaryl metabolism and to gain a more complete understanding of the general process of conjugative metabolism.

Methods

Treatment of animals - Rats weighing ca. 200 g were administered oral doses of 1 uCi of carbarylnaphthol-1-^{14}C or naphthol-^{14}C, each with a specific activity of 15 uCi/mmole, in 0.1 ml of corn oil. Some of the animals were treated simultaneously with 50 or 100 mg/kg of a monoamine oxidase inhibitor. Other animals were maintained on drinking water containing phenelzine, 80 mg/liter, for 25 days and then treated orally with the carbaryl-^{14}C. Actual intake of the drug was calculated from the total daily consumption of water.

Monoamine oxidase inhibitors evaluated for their effect on the excretion of carbaryl from rats were: (A) tranylcypromine (trans-2-phenyl cyclopropylamine); (B) isoniazid (isonicotinic acid hydrazide); (C) isocarboxazid (5-methyl-3-isoxazoledecarboxylic acid 2-benzylhydrazide); (D) phenelzine (2-phenylethylhydrazine); (E) harmaline (2,3-dihydro-7-methoxy-1-methyl-9-pyrido (3,4b) indole); (F) iproniazid (2-isopropyl-1-isonicotinyl hydrazine); and (G) pargyline (N-methyl-1-N-propargylbenzylamine). For all other studies reported herein, phenelzine was the only monoamine oxidase inhibitor used.

Collection and analysis of the excreta - Feces and urine were collected at 12-hr intervals following treatment of the rats and assayed immediately for radioactive content. Aliquots of urine, up to 1 ml, were counted directly, whereas feces were combusted first and aliquots of a CO_2 trap were assayed for radioactivity (Andrawes et al., 1967). Urine samples, used for more complete analysis, were frozen in plastic bags. Feces were discarded after the initial radioassay.

To extract the urine, it was diluted 2-fold with distilled water and partitioned repeatedly with diethyl ether. The 2 solvent phases were radioassayed, and the ether soluble products were analyzed further by tlc on Chromar 500 sheets (Mallinkrodt, St. Louis, Mo.). Analytical standards of carbaryl and 1-naphthol were added to the ether extract prior to spotting. The sheets were developed first in a 1:1 mixture of ether and hexane, and then in the 2nd direction in a 1:1 mixture of methylene chloride and ethyl acetate. Radioautography with Kodak no-screen medical x-ray film was used to locate radioactive areas on the Chromar sheets. Carbaryl and 1-naphthol standards were visualized by placing the sheets under UV light. Quantitation of the radioactivity on the tlc included that corresponding to carbaryl, that corresponding to 1-naphthol, and the total of all other radioactivity on the sheet.

In vitro conjugation of 1-naphthol - Conjugation of naphthol-1-^{14}C was evaluated by using rat intestinal mucosal glucuronyl transferase (Howes and Hunter, 1968). Small intestines were removed from rats immediately after they were killed, and kept cool in ice cold 0.15 M KCl. Intestines were opened, were washed thoroughly with the cold KCl solution, and the mucosa was collected by scraping with a scalpel. Enzyme preparations were obtained by homogenizing the mucosal scrapings in 0.15 M KCl buffer for 4 min. A typical reaction mixture included 0.1 uCi of naphthol-1-^{14}C, 0.1 ml of 0.1 M $MgCl_2$, 1.0 of phosphate buffer (pH 7.4), 0.5 ml (1.34 umole) of uridine diphosphoglucuronic acid, potassium salt (Nutritional Biochemicals), and 2.5 ml of the enzyme. Reactions were run at 37°C in a metabolic shaker for 2 hr. The reaction mixture was extracted twice with ether and aliquots of both the organic and aqueous layers were radioassayed. The percent increase or decrease in the radioactivity partitioning into the aqueous phase was calculated with reference to the presence of the monoamine oxidase inhibitor.

Radioassay - A Packard Tri-Carb Model 3380/544 liquid scintillation counter was used for all quantitative radioassays. For counting up to 0.4 ml of water and up to 1.0 ml of organic solvents, the scintillation mixture consisted of a 2:1 toluenemethylcellusolve mixture containing 5 g/liter PPO, 0.1 g/liter POPOP, and 60 g/liter of naphthalene. Scintillation grade PPO and POPOP were used, although Fisher-purified grade solvents and naphthalene proved equal in performance to more expensive materials. Fifteen ml of the scintillation mixture was used in each counting vial. For aqueous samples containing low radioactivity, aliquots up to 1.0 ml were radioassayed in 10 ml of Aquasol R (New England Nuclear, Boston). Background counts were determined by counting aliquots of identical size derived from untreated animals.

Results and Discussion

Excretion - The rate of excretion of an oral dose of carbaryl-^{14}C from rats was reduced by the simultaneous administration of a monoamine oxidase inhibitor (Table 45). Tranylcypromine reduced the rate of excretion to a greater degree than did the other drugs when given at a dose of 50 mg/kg. Only 33% of the carbaryl-^{14}C dose was eliminated in the urine by 48 hr after treatment, whereas animals which did not receive a monoamine oxidase inhibitor excreted 71% of the dose in the urine during the same period after treatment.

Low excretion of the carbaryl-^{14}C dose in the feces of the tranylcypromine-treated rats, 0.4% of the dose, was caused in part by reduction in the total feces excreted by the animals following treatment with the drug. With tranylcypromine, animals treated with 50 mg/kg were hyperactive for 3-6 hr after the inhibitor was administered, and they showed a reduced food intake for ca. 36 hr. Animals treated with 100 mg/kg of tranylcypromine died within 12 hr. This was the only drug tested which caused death of rats treated at the 100 mg/kg level. Higher doses of the other monoamine oxidase inhibitors simply enhanced their effects on excretion (Table 45).

The effect of the other monoamine oxidase inhibitors on carbaryl-^{14}C excretion from rats was not so profound as with tranylcypromine. Isoniazid and isocarboxazid reduced overall excretion by ca. 30%, phenelzine and harmaline by ca. 20%, and iproniazid and pargyline by 10-15%. The relative effect of the drugs on carbaryl-^{14}C excretion by rats was vastly different from their effect on the in vitro metabolism of this carbamate by a 15,000g soluble fraction of rat liver (Culver et al. 1970). In that study, phenelzine and pargyline were better inhibitors of carbaryl metabolism than tranylcypromine. Phenelzine appeared to have the greatest effect on in vitro and in vivo metabolism of carbaryl. Because of this and because rats treated at 50 mg/kg with phenelzine showed no toxic symptoms at all, this monoamine oxidase inhibitor was selected for additional study.

The data in Table 45 are condensed so that the effects of the monoamine oxidase inhibitors on carbaryl can be compared readily. Actually, excreta of all treated rats were collected every 12 hr and radioassayed separately. Complete data are not presented, because the pattern of elimination was the same with each drug, and variation existed only in magnitude of effect.

Chronic exposure of phenelzine to rats for 25 days by dissolving the monoamine oxidase inhibitor in drinking water reduced the rate of excretion of a single oral dose of carbaryl-^{14}C (Table 46). The greatest reduction in excretion of the carbamate occurred during the 1st 24 hr. Total excretion of the carbaryl-^{14}C dose was about the same in the phenelzine-treated and control animals. These tests indicate that chronic exposure to the monoamine oxidase inhibitors could alter normal carbaryl metabolism and excretion in mammals. However, the level of phenelzine exposure would have to be rather excessive, since adding it to the drinking water of rats at 8 mg/liter for 25 days had only a very slight effect on the fate of the carbaryl-^{14}C.

Nature of metabolites - Comparing the chemical nature of carbaryl-^{14}C equivalents in the urine of phenelzine-treated and untreated rats gave some insight into why monoamine oxidase inhibitors decreased the rate of carbaryl excretion (Table 47). Only 48% of the carbaryl dose were eliminated as conjugates in the urine of rats receiving 50 mg/kg phenelzine. Without phenelzine, 64% of the dose were present in the 48-hr urine as water-soluble conjugates. This decrease in conjugation was coincident with an increase in 1-naphthol and with a decrease in other organo-soluble metabolites which resulted from oxidative metabolism (Dorough and Casida, 1964).

It appears, then, that both oxidative and conjugative mechanisms of metabolism were affected by the monoamine oxidase inhibitor. This is the same conclusion drawn by the in vitro studies of Culver et al. (1970). Increase in 1-naphthol was a predictable occurrence, since the efficiency of conjugation was lessened considerably. Likewise, total excretion of carbaryl-^{14}C equivalents was less because the carbamate and its metabolites were not converted to polar conjugates as rapidly in phenelzine-treated animals.

The potential is clear for increasing the quantity of toxic carbamate material in the animal's body because of continued combined exposure of the insecticides and drugs. However, the indication that certain foreign compounds may interfere effectively with conjugative mechanisms could prove beneficial rather than harmful. For example, compounds which would selectively inhibit conjugation in insects or in resistant insects but not susceptible ones would reduce the hazard of the chemical to mammals by enhancing the toxicity of the material to the pest species. Existence of such compounds is conjecture at the present time, however.

Because 1-naphthol can be conjugated directly, without first having to undergo some type of metabolism as is the case for carbaryl, it was administered orally to rats, and the effects of phenelzine on its excretion and conjugation were determined. This monoamine oxidase inhibitor only slightly reduced the excretion rate of 1-naphthol from the animals (Table 48). However, phenelzine did reduce the amount of 1-naphthol excreted in urine as water-soluble conjugates. Cumulatively, conjugation was decreased by ca. 20% during the 48-hr period after treatment as compared with animals which did not receive the phenelzine treatment. These data prove that in vivo conjugation is inhibited by the monoamine oxidase inhibitors as was indicated by the in vitro studies of Culver et al. (1970).

Having established that conjugation could be specifically inhibited, attempts were made to use an in vitro system for detailed studies of conjugative metabolism and to determine the effects of various chemicals on this metabolism mechanism. Our preliminary data show that a rat-intestine enzyme system fortified with UDPGA will conjugate 1-naphthol but will not in any manner metabolize the carbamate, carbaryl. Therefore, this system will be quite useful in studying conjugation mechanism independently of other types of biochemical reactions.

Of 3 monoamine oxidase inhibitors evaluated for in vitro conjugation of 1-naphthol, phenelzine was the most effective. This material inhibited almost 65% of the naphthol conjugated in the absence of the monoamine oxidase inhibitors. This degree of inhibition was nearly twice that reported for the inhibition of 1-naphthol conjugation by a $NADPH_2$-fortified rat liver enzyme system (Culver et al. 1970). Tranylcypromine and isoniazid were equally effective in inhibiting the conjugation by both the $NADPA_2$- and UDPGA-fortified enzyme systems.

Studies are continuing on the refinement of the in vitro conjugation system and on evaluating the importance of conjugation in the action of pesticides and other chemicals.

TABLE 45. EFFECT OF SEVERAL MONOAMINE OXIDASE INHIBITORS (MAOI) ON THE EXCRETION OF A SINGLE ORAL DOSE OF CARBARYL NAPHTHYL-^{14}C BY RATS[a]

	% of administered radioactivity eliminated by 48 hr		
MAOI	Urine	Feces	Total
Control	70.8	8.7	79.5
Tranylcypromine	32.8	0.4	33.2
Isoniazid	47.0	1.4	48.4
Isocarboxazid	49.9	4.0	53.9
Phenelzine	56.1	6.9	63.0
Harmaline	59.9	3.5	63.4
Iproniazid	60.7	4.8	65.5
Pargyline	68.8	3.0	71.8

[a] MAOI administered at a dose of 50 mg/kg simultaneously with carbaryl.

TABLE 46. FATE OF A SINGLE ORAL DOSE OF CARBARYL-^{14}C IN RATS WHICH HAD RECEIVED PHENELZINE IN THE DRINKING WATER FOR 25 DAYS[a]

Time after carbaryl treatment	% of administered radioactivity eliminated		
	Urine	Feces	Total
12 hr			
Control	61.2	1.8	63.0
Treated	47.3	0.2	47.5
24 hr			
Control	74.6	3.2	77.8
Treated	59.4	0.9	60.3
48 hr			
Control	75.7	5.8	81.5
Treated	74.1	4.0	78.1

[a] Phenelzine in drinking water at 80 mg/liter; avg. daily intake of 1.3 mg/day.

TABLE 47. NATURE OF CARBARYL-^{14}C METABOLITES IN URINE OF NORMAL AND PHENELZINE-TREATED RATS[a]

	% of administered carbaryl dose in	
Metabolite	Normal rats	Treated rats
Carbaryl	0.4	0.1
Naphthol	1.3	4.8
Organo-solubles[b]	9.0	3.3
Conjugates[c]	63.8	47.9
Total	74.5	56.1

[a] Analysis of urine from rats collected 48 hr after treatment with a single oral dose of carbaryl-^{14}C or with carbaryl-^{14}C + phenelzine at a dose of 50 mg/kg.

[b] Combined organo-soluble metabolites, excluding carbaryl and naphthol.

[c] Total water-soluble metabolites.

TABLE 48. METABOLISM AND EXCRETION OF NAPHTHOL-^{14}C WHEN ADMINISTERED TO RATS SIMULTANEOUSLY WITH AN ORAL DOSE (50 MG/KG) OF PHENELZINE

Time after treatment	Cumulative % of dose in the Urine	Feces	Total	% of dose conjugated[a]
12 hr				
Control	39.2	0.4	39.6	27.8
Treated	43.8	0.9	44.7	39.2
24 hr				
Control	56.2	1.3	57.5	55.4
Treated	50.9	2.0	52.9	46.1
48 hr				
Control	71.4	4.8	76.2	70.0
Treated	59.8	4.9	64.7	48.6

[a] These values reflect the percentage of the dose present in the urine as water-soluble metabolites.

Influence of Selected Insecticides on Carbaryl Metabolism

Investigating the metabolism of a compound in a complex environment containing other compounds may aid in understanding the ways various compounds affect one another in the biotransformation processes in organisms. This was attempted in the present investigation to gain some knowledge of changes in the metabolism of a carbamate insecticide in the rats which also were treated with other types of insecticides.

Methods and Materials

Standards - Carbaryl-1-naphthyl-^{14}C (specific activity, 6.56 mc/mM) was supplied by Union Carbide Corporation, Olefins Division, South Charleston, West Virginia. Pure 1-naphthol-^{14}C (15.2 mc/mM) was purchased from Amersham Searle (Chicago, Ill). Several analogs of carbaryl considered as possible metabolites were provided by Union Carbide Corporation.

Analytical standards of aldrin, dieldrin, heptachlor, and heptachlor epoxide were purchased from Unilab Research Corporation, Berkeley, California. The purity of each chemical was over 99% as determined by gas chromatography.

Technical insecticidal chemicals used were carbofuran, aldrin, heptachlor, DDT, Ruelene, and coumaphos. All were purchased from City Chemical Corporation, New York.

Treatment and sampling - Rats were treated with a single dose or multiple daily doses of insecticides via various routes and for various periods.

For investigating the effect of a carbamate or an organophosphate treatment on the metabolism of carbaryl in the rat, three groups of rats (3 rats in each group) were administered orally a single dose of carbaryl-^{14}C (50 mg/kg) only, and in combination with carbofuran (0.5 mg/kg) or Ruelene (4.6 mg/kg). The insecticides were administered in corn oil. The rats were placed in metabolism cages where the urine and feces were collected at 12-hr intervals for 7 days and radioassayed. The urinary metabolites of carbaryl were analyzed using thin layer chromatography.

For evaluating the effects of multiple daily doses of carbaryl and a carbamate or an organophosphate on the metabolism of carbaryl-^{14}C, rats were administered orally a daily dose of carbaryl-^{14}C 260,000 dpm, (50 mg/kg) for 4 days. Concurrently, rats were continuously provided insecticide-free rat chow or rat chow fortified with carbofuran (5 ppm) or Ruelene (46 ppm) or coumaphos (15 ppm). The animals were maintained individually in metabolism cages and provided the diets and water for a period of 2 weeks. Urine and feces were collected at 24-hr intervals for 2 weeks, radioassayed and the nature of metabolites determined.

The effects of injecting rats with DDT, carbaryl, carbofuran and Ruelene on various enzyme activities in the liver and kidney microsomes were investigated. Rats were administered intraperitoneally a daily dose of DDT (25 mg/kg) or carbaryl (50 mg/kg) or a mixture of carbaryl plus DDT (50 mg and 25 mg/kg) or carbofuran (0.5 mg/kg) for up to 5 days. Three rats were used for each treatment and for the control. Insecticides were dissolved in corn oil and the control rats were given corn oil at the same volume as for the treated rats.

Rats also were given rat chow containing DDT (500 ppm), carbaryl (1,000 ppm), DDT plus carbaryl (500 + 1,000 ppm) or carbofuran (5 ppm) for a period of 40 days. Animals from these treatments were given a single oral dose of carbaryl-^{14}C 24 hours after the last exposure to the insecticide via injection or in the diet. All rats, upon completion of the insecticide administrations, were weighed and sacrificed. The livers, kidneys and spleen were removed for assays of microsome enzyme activities.

For investigating the effect of pre-exposure to DDT on the metabolism of 1-naphthol-^{14}C in the rat, three rats were given intraperitoneally a daily dose of DDT (50 mg/kg) for 5 days and a single oral dose of 1-naphthol-^{14}C 24 hours later. Urine and feces were collected for 48 hours after treatments and radioassayed. The metabolites of carbaryl and naphthol in the urine were analyzed using thin layer chromatography.

Analysis of urine - Urines from rats collected for 60 hours after the respective treatments were analyzed. The sample was added to an equal amount of distilled water, mixed well and transferred to a 125-ml separatory funnel. It was saturated with sodium chloride and then directly extracted 5 times with a triple volume of a 3:1 mixture of chloroform-acetonitrile. The wall of the separatory funnel was rinsed with 5 ml of distilled water and extracted twice with the chloroform-acetonitrile mixture. The organosoluble and aqueous phase were separated and radioassayed.

The chloroform-acetonitrile extract (organosoluble phase) was dried with anhydrous sodium sulfate and filtered. The extract was concentrated and spotted on a 8 x 8 inch ChromAR 500 tlc sheet for analysis of the organosoluble metabolites.

The aqueous phase obtained after extraction of the urine sample was filtered and concentrated to 1 to 2 ml in a rotary evaporator, then transferred to a 15-ml centrifuge tube. After concentrating to about 0.5 ml under a stream of air, the final concentrate was spotted as a band on a 8 x 8 inch of ChromAR 500 sheet for separating the water soluble metabolites.

Ascending thin layer chromatography using 8 x 8 inch ChromAR 500 sheets (Mallinckrodt Chemical Works, St. Louis, Missouri) was utilized for resolving carbaryl and its metabolites. For separation of organosoluble carbaryl metabolites, the ChromAR sheet was developed two-dimensionally, first in a 4 to 1 ether-hexane mixture then in a 3 to 1 chloroform-acetonitrile mixture. For separation of water soluble metabolites, the tlc was developed in a 2:1 mixture of ethyl acetate and isopropanol.

Radioactive areas on the chromatograms were located by radioautography after exposure to x-ray film for 3 to 5 days. The radioactive area of the tlc was extracted with ether, while the water solubles were extracted with distilled water. The radioactivity in each extract was determined.

Metabolite identification - The identity of each of the carbaryl metabolites was tentatively determined by cochromatography with standard carbaryl and its derivatives. The water soluble metabolites of carbaryl were also identified according to their aglycones after acid hydrolysis. For convenience, carbaryl and 1-naphthol were quantitated separately, while the other metabolites were combined and referred to as extractable carbamate metabolites (Dorough and Casida, 1964). The unhydrolyzed material was considered as the unknown water soluble metabolites.

Enzyme studies - Upon completion of insecticide administrations, the animals were sacrificed and their livers, and kidneys immediately excised and placed in ice cold Tris-HCl buffer (0.05 M, pH 7.0) containing 8.6% sucrose. The liver or kidney was macerated and homogenized in Tris-HCl buffer (50% by weight). The homogenate was centrifuged at 15,000g for 30 minutes at 3°C in an ultracentrifuge (Beckman L2-65B). The supernatant was further centrifuged at 105,000g for 60 minutes. The final pellet was washed once with a 1.2% KCl solution, centrifuged and resuspended thoroughly in Tris-HCl buffer (0.05 M, pH 7.0) so that 1 ml of buffer contained 500 mg tissue equivalents. A portion of the 105,000g preparation was used immediately for oxidative enzyme assay while the remainder was stored at -20°C for conjugative enzyme assay.

Microsomal oxidative and conjugative enzyme activities were determined by measuring the rates of TPNH oxidation, epoxidation of aldrin and heptachlor, total metabolism of 1-naphthyl-^{14}C-carbaryl, and glucuronide conjugation of naphthol-^{14}C. The method for carbaryl metabolism was similar to that reported by Dorough et al., (1964) and Oonnithan et al., (1968). The microsomal conjugating enzyme activity was determined by the in vitro method of Mehendale and Dorough (1971) while the TPNH oxidase activity was measured according to Hart and Fouts (1965). Epoxidase activity was determined by measuring the epoxidation of aldrin to dieldrin and of heptachlor to its epoxide. For these studies, a Varian Aerograph gas chromatograph was employed. It was equipped with an electron capture detector and nitrogen as the carrier gas. The glass column, 6' x 1/8", contained 4% SE on Anakrom ABS, 80/90 mesh, and was operated at a column temperature of 200°C, detector temperature 215°C, injector temperature 205°C and inlet pressure of 10 psi.

Lowry's method (1951) was used for protein determination with the standard curve constructed using bovine serum albumin crystalline (Nutritional Biochemical Corporation, Cleveland, Ohio).

Results and Discussion

The urinary and fecal excretion patterns of an orally administered dose of carbaryl-^{14}C equivalents to rats are given in Table 49. Statistical analyses revealed that there were no significant differences in the excretion when carbaryl was administered alone or in combination with Ruelene. However, when carbofuran was administered along with carbaryl-^{14}C the excretion of carbaryl equivalents in the urine was significantly reduced. The depression was most evident during the first 24 hours after the treatment. The average percentage reduction of excretion of carbaryl equivalents caused by administration of carbofuran was 12.2% in the urine and 3.4% in the feces over a 7-day period.

The excretion of carbaryl equivalents in urine and feces of rats following 4 daily oral doses of carbaryl-^{14}C given at 24-hr intervals was quite different from that following a single oral dose of the carbaryl (Table 50). Treatment with the 4 consecutive doses of carbaryl reduced the excretion of carbaryl equivalents in the urine and feces by about

20% during the first 72 hours after the first dose compared to that from rats dosed with a single oral dose of carbaryl. Similarly, a 12% decrease after 72 hours was exhibited by animals which received daily oral doses of carbaryl for 4 days while on a diet containing 5 ppm of carbofuran. Conversely, excretion of carbaryl equivalents by rats receiving the daily doses of carbaryl while on a diet containing Ruelene was increased as compared with that when animals were given carbaryl alone. The effect of Ruelene on increasing the excretion rate of carbaryl equivalents was not as remarkable as that of carbofuran on reducing the excretion rate of carbaryl equivalents. Ten days after the first dose of carbaryl, the percentage excretion of carbaryl equivalents in urine plus feces was 90% for animals receiving 4 doses of carbaryl alone, 86% for those which received carbaryl and fed on the carbofuran diet, and almost 100% for animals receiving carbaryl and feeding diets containing either Ruelene or coumaphos.

It was possible that the reduction in the excretion of carbaryl equivalents after treatment with repeated doses of carbaryl or carbofuran was caused by an overloading and increased toxicity of the carbamates to the animals. This was supported by the finding that during the period of consecutive dosing of carbaryl the excretion of carbaryl equivalents was slowed. This was also suggested by Dorough (1967) who reported that increasing the carbaryl dose to cows decreased slightly the excretion of carbaryl in urine and feces.

The slight increase in the excretion rate of carbaryl equivalents in the urine of rats continuously feeding diet containing Ruelene indicated that there was an acceleration in the metabolic rate of carbaryl in the animal after repeated doses with Ruelene in the diet. Stimulation of biotransformation of carbaryl by Ruelene in the rat could be possible.

Partitioning the radioactive metabolites of carbaryl in the urine of rats treated with a single oral dose of carbaryl alone or in combination with carbofuran or Ruelene showed that the percentage distribution of metabolites between chloroform and water was dissimilar for all animals (Table 51). Of the total radioactivity in the 0-12 hour urine, approximately 15% was organo-extractable for animals treated with carbaryl plus carbofuran while 22% was organo-extractable for animals treated with carbaryl plus Ruelene. For rats treated with carbaryl only the comparable value was 20%. As expected from the excretion data (Table 49), the carbaryl-carbofuran treatment resulted in less total metabolites, both organo-extractable and water soluble, in the urine when expressed on a "percent of dose" basis.

Route, timing of administration and dosage levels may effect different responses of organisms to a toxic compound; it was therefore decided to investigate the effects of preadministrations with insecticides on the metabolism of carbaryl in rats. For *in vitro* liver microsomal enzyme activities were evaluated for any induction or inhibition caused by exposure to the insecticides.

The liver weight of rats which received 500 ppm p,p'-DDT in the diet for 40 days increased 30% compared to the livers of control animals (Table 51). There was no change in the weight of the kidney of treated rats. The results were in agreement with those of Wasserman (1969) who showed that rats receiving 200 ppm of p,p'-DDT in the drinking water for 35 days showed an increase in the liver weight by 14% compared with that of the control animals.

Carbaryl given intraperitoneally or in the rat feed, or carbofuran given intraperitoneally, did not change the weights of the liver and the kidney.

Accompanying the increase in the liver weight after DDT treatment was an increase in the liver microsome protein content (Table 52). The rat liver microsome protein content increased 53% after intraperitoneal DDT treatment for 5 days (25 mg/kg daily) compared to the liver microsome protein content in the DDT-free rats. The protein content in the kidney microsomes was not altered by DDT. The increase in the liver microsome protein content after DDT treatment could be the result of induction of protein biosynthesis by DDT in rats as suggested by a report that DDT at 0.5 ppm in Hela S cell culture medium increased the incorporation of ^{14}C-leucine into cellular protein (Chung et al., 1967).

Carbaryl or carbofuran when given intraperitoneally, 50 mg/kg daily and 0.5 mg/kg daily, respectively for 5 days, caused a decrease by 30% of the liver microsome protein content in the treated rats. The result indicates that carbaryl and carbofuran inhibited protein biosynthesis in rats.

The change in the liver or the kidney microsome protein content correlated with its metabolizing enzyme activities. DDT treatment caused significant increases in the liver or kidney microsomal enzyme activities for TPNH oxidation, epoxidation of heptachlor and aldrin and total metabolism of carbaryl (Table 53). At 500 ppm for 40 days, DDT caused a 133% increase in the TPNH oxidase activity; 1270% increase for epoxidation of heptachlor; and over 800% for carbaryl metabolism. DDT, given intraperitoneally at 25 mg/kg daily for 5 days, also caused an increase in the liver microsome enzyme activity for TPNH oxidation (Table 54). However, treatment with DDT, at 500 ppm for 40 days, did not enhance the *in vitro* liver microsomal enzyme activity for glucuronidation of 1-naphthol.

Carbaryl, given in the feed (1,000 ppm for 40 days) or given intraperitoneally (50 mg per kg for 5 days), did not cause significant increases in the liver or kidney microsome enzyme activities for TPNH oxidation, heptachlor or aldrin epoxidation, carbaryl metabolism and 1-naphthol conjugation (Tables 53 and 54).

Pre-exposure to DDT (25 mg/kg daily) for 3 days induced slight increases in content of the organosoluble and water soluble metabolites of carbaryl in the 48-hr urine of rats (Table 55). DDT preadministration

(50 mg/kg daily) for 5 days also enhanced the urinary excretion of 1-naphthyl glucuronide and 1- naphthyl sulfate when the rats were treated with naphthol-^{14}C (Table 56).

The increases in microsomal enzyme activities for TPNH oxidation, heptachlor or aldrin epoxidation, carbaryl hydroxylation and conjugation in the rat after DDT pretreatments demonstrated a positive induction or stimulation by DDT of the metabolizing enzyme systems in the mammal. Increased enzyme activities were probably the result of induction on syntheses of enzyme proteins in the animal. These results were generally in agreement with reports in the literature. For instance, Hart and Fouts (1965) observed an increase of liver microsomal TPNH oxidase activity in rats receiving chlordane. Gillett et al., (1968) reported that the epoxidation of aldrin to dieldrin and heptachlor to its epoxide were greatly enhanced by microsomal preparation from rats which received DDT.

The failure of carbaryl or carbofuran to induce significant increases in the liver or kidney microsome enzyme activities for oxidation of TPNH, heptachlor and carbaryl, and for conjugation of carbaryl metabolites might be the result of inhibition of the microsomal protein biosynthesis in the liver by the carbamate. Furthermore, carbamates are generally unstable and easily metabolized in animals, and thus might not persist in the body long enough to produce an effect. Therefore, the carbamate did not appear as a microsome metabolizing enzyme inducer.

Preadministration of Ruelene (4.6 mg/kg daily) for 3 days slightly increased the urinary organosoluble and water soluble carbaryl metabolites in the treated rats. Simultaneous administration of Ruelene in the feed (46 ppm) also slightly hastened the excretion of carbaryl equivalents in the urine as mentioned earlier. This suggests that Ruelene at the dose applied was possibly a weak microsomal enzyme inducer.

In order to stimulate significantly the liver microsomal enzyme activities, it may require increases in the dose rates of the carbamates and/or the organophosphorus insecticides. As Fouts (1970) suggested, test substances that are rapidly excreted, metabolized or sequestered in non-hepatic tissues are not usually good enzyme inducers unless they are given repeatedly or at a very high dose.

TABLE 49. THE EFFECTS OF CARBOFURAN AND RUELENE ON THE EXCRETION OF CARBARYL-^{14}C ADMINISTERED TO RATS AS A SINGLE ORAL DOSE[a]

Hours after	Cumulative percent of dose after indicated treatment[b]					
	Carbaryl		Carbaryl + Carbofuran		Carbaryl + Ruelene	
treatment	Urine	Feces	Urine	Feces	Urine	Feces
12	28.8	1.3	21.5	0.8	28.8	0.8
24	67.3	4.0	51.7*	2.6	61.8	3.0
48	75.0	7.8	60.9*	4.8	77.5	4.7
72	76.6	9.4	63.4*	5.9	77.5	5.9
96	77.5	9.8	64.8*	6.4	78.0	6.6
120	77.6	9.9	65.8*	6.4	79.2	6.6
168	79.7	9.9	67.5*	6.5	80.2	6.7
Avg. SD	5.6	1.3	4.2	1.3	6.9	1.6

[a] Naphthyl-1-carbaryl-^{14}C dose of 50 mg/kg; carbofuran, 0.5 mg/kg, or Ruelene, 4.6 mg/kg, administered simultaneously with carbaryl.

[b] Asterisk indicates a significant difference at the 5% level from the carbaryl treatment as determined with t-test.

TABLE 50. EXCRETORY PATTERN OF CARBARYL WHEN ADMINISTERED AS A DAILY SINGLE ORAL DOSE TO RATS FOR 4 DAYS WHILE FED A NORMAL RATION AND WHEN FED A DIET CONTAINING CARBOFURAN, RUELENE OR COUMAPHOS[a]

Hours after first carbaryl treatment	Cumulative percent of carbaryl dose when diet contained[b]							
	Control		Carbaryl + Carbofuran		Carbaryl + Ruelene		Carbaryl + Coumaphos	
	Urine	Feces	Urine	Feces	Urine	Feces	Urine	Feces
12	22.7	0.6	29.9	0.9	36.2	0.7	32.3	0.8
24	30.3	1.0	30.7	1.1	48.0*	0.8	42.3	0.9
72	58.7	7.4	49.6	7.6	74.5*	9.7	64.0	6.3
96	66.4	9.0	55.6*	8.7	89.0*	9.8	69.4	7.0
120	80.7	9.2	72.0*	9.0	90.2*	9.8	85.9	9.3
168	87.0	10.8	75.4*	9.6	90.2	9.8	86.1	10.0
Avg. SD	4.5	1.4	1.5	1.4	7.0	1.2	6.4	1.4

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[a] Naphthyl-1-carbaryl-^{14}C dose of 50 mg/kg; levels in the diet for carbofuran, Ruelene and coumaphos were 5, 46, and 15 ppm, respectively. Insecticide fortified diets were provided from the time of the first carbaryl treatment until termination of experiment.

[b] Asterisk indicates a significant difference at the 5% level from the carbaryl treatment as determined with t-test.

TABLE 51. NATURE OF RADIOACTIVITY IN THE URINE OF RATS TREATED WITH A SINGLE ORAL DOSE OF NAPHTHYL-1-CARBARYL-^{14}C ALONE, AND WHEN ADMINISTERED SIMULTANEOUSLY WITH EITHER CARBOFURAN OR RUELENE[a]

Nature of metabolites	Percent of dose when treated with:					
	Carbaryl		Carbaryl + Carbofuran		Carbaryl + Ruelene	
	0-12 hr	12-48 hr	0-12 hr	12-48 hr	0-12 hr	12-48 hr
Organoextractable[b]	5.8	7.8	3.2	7.3	6.4	11.1
carbamate metabolites	1.9	2.5	0.9	3.7	2.2	2.8
Hydrolysis Products	3.2	4.2	2.2	2.9	3.4	7.4
Unknowns	0.7	1.1	0.1	0.7	0.8	0.9
Watersolubles[c]	23.0	38.4	18.3	28.5	22.4	32.6
Carbamate metabolites	2.5	1.1	1.4	2.2	2.4	2.0
Hydrolysis Products	2.9	1.4	0.6	2.7	2.9	2.6
1-Naphthyl sulfate	5.2	8.3	3.7	5.1	4.8	6.8
Unknowns	12.4	27.6	12.6	18.5	12.3	21.2

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[a] Dosage rates same as given in Table 49.

[b] Metabolites in urine which were extractable into organic solvent. Carbamate metabolites refer to those materials with the carbamate ester linkage still intact.

[c] Nature of metabolites based on aglycones produced by acid treatment of the watersolubles. Unknowns include those materials remaining in the water after acid treatment.

TABLE 52. WEIGHT AND MICROSOMAL PROTEIN CONTENT OF LIVERS FROM RATS EXPOSED TO CARBARYL, CARBOFURAN AND DDT

Treatment method and duration	Liver weights, percent of body weight[a]	Protein content, mg protein per gram of liver[a]
IP injections for 5 days		
Control (corn oil)	4.4 ± 0.3	20.9 ± 2.0
Carbaryl, 50 mg/kg/day	3.5 ± 0.2*	15.9 ± 1.8*
Carbofuran, 0.5 mg/kg/day	4.0 ± 0.3	15.4 ± 1.7*
DDT, 25 mg/kg/day	4.6 ± 0.5	32.4 ± 5.1*
DDT + Carbaryl	4.1 ± 0.4	19.3 ± 3.7
In diet for 40 days		
Control	3.7 ± 0.3	17.7 ± 1.4
Carbaryl, 1000 ppm	3.2 ± 0.1	20.9 ± 1.4
DDT, 500 ppm	4.8 ± 0.3*	19.3 ± 1.3
DDT + Carbaryl	4.0 ± 0.1	18.2 ± 1.4

[a] Asterisk indicates a significant difference at the 1% level compared to the control animals as determined with t-test.

TABLE 53. IN VIVO METABOLIC ACTIVITY IN THE MICROSOMAL FRACTIONS OF LIVER AND KIDNEY OF RATS FED DIETS CONTAINING CARBARYL, AND DDT FOR 40 DAYS

	Metabolic activity[b]			
Treatment and rate[a]	% TPNH oxidation	% Heptachlor epoxidation	% Carbaryl metabolism	% Naphthol conjugation
		Liver		
Control	3.5 ± 0.16	3.7 ± 0.04	3.6 ± 0.05	81.9 ± 1.2
Carbaryl, 1000 ppm	4.1 ± 0.15	4.8 ± 0.25	6.3 ± 0.18	93.4 ± 0.9
DDT, 500 ppm	8.0 ± 0.26*	50.9 ± 3.87*	33.2 ± 1.57*	86.5 ± 2.3
DDT + Carbaryl	5.8 ± 0.85*	59.7 ± 0.25	28.5 ± 3.71*	86.4 ± 1.8
		Kidney		
Control	1.8 ± 0.90	0	0	81.9 ± 5.5
Carbaryl, 1000 ppm	3.5 ± 0.53*	0	0.7 ± 0.05	95.0 ± 0.1
DDT, 500 ppm	4.6 ± 0.90*	18.3 ± 0.3*	0.4 ± 0.04	89.3 ± 0.2
DDT + Carbaryl	4.3 ± 0.37*	17.4 ± 0.8*	0.8 ± 0.02	87.4 ± 0.5

[a] Treatment rates for DDT + carbaryl were the same as individual rates shown.

[b] Asterisk indicates a significant difference at the 5% level compared to the controls as determined with t- test.

TABLE 54. TPNH OXIDASE ACTIVITY IN THE MICROSOMAL FRACTION OF LIVERS FROM RATS TREATED INTRAPERITONEALLY WITH CARBARYL AND DDT DAILY FOR UP TO 5 DAYS

Treatment and daily dose	Percent TPNH oxidized/days on treatment[a]		
	1	3	5
Control	2.5 ± 0.23	3.6 ± 0.16	4.2 ± 0.16
Carbaryl, 50 mg/kg	3.0 ± 0.37	3.6 ± 0.05	3.1 ± 0.53*
DDT, 25 mg/kg	3.1 ± 0.42	4.1 ± 0.01	11.1 ± 0.37*
DDT + Carbaryl	3.4 ± 0.10	3.0 ± 0.58	4.2 ± 0.53

[a] Asterisk indicates a significant difference at the 1% level from the controls as determined with t-test.

TABLE 55. THE EFFECT OF PRE-EXPOSURE TO DDT (IP, 25 MG/KG/DAY) FOR 3 DA.S ON THE NATURE AND MAGNITUDE OF CARBARYL METABOLITES IN THE URINE OF RATS TREATED WITH A SINGLE ORAL DOSE OF NAPHTHYL-1-CARBARYL-^{14}C[a]

Nature of metabolites	Percent of dose and (% of total urine content)	
	Control rats	DDT-treated rats
Organoextractable[b]	7.7 (10.6)	11.3 (13.4)
Carbamate metabolites	3.9 (5.4)	5.7 (6.8)
Hydrolysis products	0.4 (0.5)	1.1 (1.3)
Unknowns	3.4 (4.7)	4.5 (5.3)
Watersoluble[c]	64.8 (89.4)	73.2 (86.6)

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[a] Naphthyl-1-carbaryl-^{14}C dose of 50 mg/kg given 24 hours after the last injection of insecticide.

[b] Metabolites in urine which were extractable into organic solvent. Carbamate metabolites refer to those materials with the carbamate ester linkage intact.

[c] Metabolites remaining in the water phase after extraction with organic solvent.

TABLE 56. EXCRETION AND METABOLISM OF A SINGLE ORAL DOSE OF NAPHTHOL-1-^{14}C BY RATS GIVEN DAILY INJECTIONS OF DDT, 50 MG/KG FOR 5 DAYS

Route of excretion/nature of urine metabolites[a]	Percent of naphthol-1-^{14}C administered	
	Control rats	DDT-treated rats
Excretion		
Feces	1.14 ± 0.23	1.20 ± 0.23
Urine	58.43 ± 6.52	66.41 ± 7.10
Urine metabolites		
Total organosolubles	8.18	11.65
Total watersolubles	50.25	54.76
1-naphthyl glucuronide	15.24	29.51
1-naphthyl sulfate	0.39	13.91
Unknowns[b]	34.62	11.34

[a] Based on collection of total excreta during first 24 hours after treatment with naphthol.

[b] Materials remaining at the origin after tlc analysis.

CONJUGATION

Mechanism in Rats

Insecticidal carbamates may be subjected to conjugative metabolism even more extensively than other groups of insecticides (Dorough, 1970). In fact, some are almost quantitatively transformed to, and excreted as, conjugate materials. Table 57 lists several carbamate insecticides which were voided primarily as conjugate materials by rats. Most of these metabolites were glucuronides or ethereal sulfates as evidenced either by comparison with chemically synthesized standards or by subjection of these materials to specific hydrolytic enzymes.

Formation of glycosides is the predominant form of conjugation in animals, insects, and plants. In animals, glucuronides are formed by a reaction catalyzed by uridine diphosphoglucuronyl transferase (EC 2.4.1.17) and is characterized by the utilization of uridine diphosphoglucuronic acid. Present evidence indicates that the enzyme activity is associated in the particulate fractions of cell-free preparations (Parke, 1968). Different substrates are conjugated with glucuronic acid by different enzymes.

Much of the recent literature in the area of insecticide toxicology and metabolism deals with the initial oxidative modification of the insecticide chemical. This is understandable since many insecticides are anticholinesterase agents and consideration of the reactions responsible for the initial metabolism, and its effect on anticholinesterase activity, are of obvious toxicological significance. Also, biochemical oxidation was envisioned as a target to control the toxicity of this type of compound. For example, it has been shown that the mechanism of action of insecticide synergists involves their ability to inhibit the microsomal oxidative enzymes (Casida, 1970). These very important consequences of insecticide bioalterations may not be restricted to the oxidative phase of metabolism.

It is possible that the action of insecticide synergists, the mechanism of insect resistance, and the selective nature of some insecticides could be related to conjugative metabolism. The possibility that such could be the case had been suggested earlier (Mehendale and Dorough, 1971). While one study (Boose and Terriere, 1967) showed no apparent increase in conjugative ability in chlorinated hydrocarbon-resistant houseflies, Culver et al., (1970), reported the inhibition of naphthol conjugation, as well as the total metabolism of carbaryl, by certain monoamine oxidase inhibitors. Later, it was demonstrated that the same drugs inhibited the conjugation of 1-naphthol by a rat intestine enzyme system (Dorough, et al. 1972). When administered orally to rats some of the drugs reduced the rate of elimination of carbaryl from the animals and caused minor changes in the nature and the quantity of individual metabolites in the excreta.

Since it was established that conjugation was a major metabolic pathway for carbamates in animals and that the pathway was sensitive to various foreign compounds, a detailed study directed toward establishing a well-defined in vitro conjugative system was conducted. The hydrolytic product of carbaryl, 1-naphthol, was selected as the substrate for studying the enzyme system responsible for glucuronidation by rat liver and intestine enzymes since it could be conjugated directly.

Methods and Materials

Substrate and enzyme - 1-Naphthol-1-^{14}C (15 mCi/mmole) was obtained from Amersham Searle Co., Des Plaines, Ill, while radiolabeled carbaryl (1-naphthyl-1-^{14}C-N-methylcarbamate) (5.6 mCi/mmole) was supplied by Union Carbide Corporation, Charleston, West Virginia.

Livers and small intestines of female albino rats weighing approximately 200 g were used as the enzyme souces in all studies. All rat tissue preparations were made so that each milliliter of buffer contained the enzyme from 250 mg of tissue. Each centrifugal fraction was tested for glucuronyl transferase activity, but only the 15,000g solubles and microsomal fractions were sufficiently active for use in routine enzyme assays.

A second source of enzyme from the rat was the small intestines. In preliminary experiments, homogenates of the mucosal scrapings from cleaned rat intestines were used as the enzyme source. Later, it was discovered that the 12,000g soluble fraction of homogenates from cleaned, whole gut tissue could be used just as effectively. Therefore, whole intestines were homogenized in 4 vol of 0.15 M KCl solution and the 12,000g soluble fraction used as the enzyme source. The enzyme activity in the 12,000g soluble fraction was increased 3-fold by adding 1% digitonin to the KCl solution.

Enzyme assay - For routine enzyme assays, 1 umole 1-naphthol-^{14}C (diluted with unlabeled 1-naphthol such that 1 umole was equivalent to 2×10^5 dpm) was introduced into a 25-ml Erlenmeyer flask and the solvent evaporated just to dryness. The other constituents were added to the flask in buffer solutions. After the flasks were transferred to a metabolic shaker with the water temperature at the desired level, the cofactor was added, and incubation time was recorded from that point. Reactions were terminated by the addition of 2.0 ml of diethyl ether to the flask.

Extraction - Unreacted 1-naphthol-^{14}C was extracted by transferring the reaction mixture to a 15-ml glass-stoppered centrifuge tube and extracting it twice with 2 vol of diethyl ether. These extractions yielded a recovery of over 95% of the free 1-naphthol and, therefore, further extraction was not necessary. After separation of the ether and water phases, the ether layer was adjusted to 10.0 ml and a 1.0 ml aliquot removed for radioassay by liquid-scintillation counting. An aliquot of 0.2 ml of the aqueous layer was radioassayed to determine the water-soluble naphthyl glucuronide formed.

Nature of the reaction products - Ether extracts were concentrated and applied to Chromar 500 thin-layer chromatographic sheets and developed two dimensionally. The first solvent system was a 5:1 mixture of hexane and ether and the second system a 2:1 mixture of methylene chloride and ethylacetate. A standard sample of 1-naphthol was used for co-chromatography.

The aqueous phase was concentrated and subjected to beta-glucuronidase for 24 hr (24), and the reaction mixture was extracted twice with 2 vol of ether. The two solvent phases were radioassayed and the ether extract examined on tlc to determine the nature of the aglycone.

Inhibition of conjugating enzyme - The chemical being tested for its effect on the conjugating enzyme systems was introduced into a reaction flask as an ether solution just after the substrate was added. The ether was evaporated using a gentle jet of air. Other constituents of the reaction mixture were added in the manner described before and the reactions carried to completion. Changes in the amount of conjugation of 1-naphthol were determined and the degree of reduction of 1-naphthol conjugation was expressed as percentage of inhibition as compared to that of a control sample.

Metabolism of carbaryl - The metabolism of carbaryl by the microsomal fraction of the rat liver was investigated. $NADPH_2$ was introduced into the flasks at the rate of 2 umoles per flask, but the other components were the same as the standard conditions for conjugation of 1-naphthol. These flasks were incubated for 2 hr to achieve maximum metabolism of carbaryl.

Epoxidation of aldrin by rat liver microsomes - To determine the relative effectiveness of certain insecticide synergists on oxidative and conjugative metabolism, a limited number of experiments were conducted on the microsomal epoxidation of aldrin. A typical reaction mixture contained the following ingredients: 20 ug of aldrin in 100 ul of methyl cellosolve; 2 umoles of $NADPH_2$ in 0.5 ml of buffer; 2.4 ml of Tris-HCl (0.05 M) buffer, pH 7.4; 1.0 ml of rat liver microsomes (250 mg tissue equivalents); and 0.1 ml of ethanol containing the desired amount of synergists. The mixtures were incubated for 1 hr at 37°C in a metabolic shaker. At the end of the incubation period, the reactions were terminated by adding 4.0 ml of a 2:3 mixture of isopropanol and hexane and then extracted twice with 2 vol of hexane. The organic solvent phase was analyzed for aldrin and dieldrin by gas chromatographic means using a Varian Aerograph Model 1700 instrument equipped with an electron-capture detector. Operating parameters were as follows: column, glass, 6 ft x 1/8 in. i.d. packed with 10% DC 200 on Anakrom ABS, 80-90 mesh; carrier gas, nitrogen, 45 ml/min.; temperature, column 195°C, injection port 200°C, detector 215°C. Retention times for aldrin and dieldrin were 3.8 and 9.6 min., repsectively.

Results and Discussion

Optimum conditions for in vitro glucuronidation - For preliminary investigations into the glucuronidation mechanisms in rats, a liver preparation commonly used for the metabolism of carbamates and other chemicals was employed (Knaak et al., 1965). This basic system was modified and refined until the optimum conditions for the glucuronidation of 1-naphthol-^{14}C by enzymes from rat liver and small intestine were established. The parameters for obtaining 1-naphthyl glucuronide in greatest quantity were essentially the same for both enzyme sources and were as follows:

Substrate	1-Naphthol-1-^{14}C, 1.0 umole
Buffer	Tris-HCl, 0.05 M, pH 7.0, 1.0 ml
Metal ion	$MgCl_2$,100 umoles in 1 ml buffer
Enzyme	250 mg tissue equivalents in 1 ml buffer
Cofactor	UDPGA, 1.4 umoles in 1 ml buffer
Incubation	37°C, for 15 min, with shaking.

Generally, the conjugating enzymes were not too sensitive to temperature variations between 35°C and 50°C. Temperatures outside this range reduced the enzyme activity considerably, especially as the incubation temperature exceeded 50°C. Careful examination of the enzyme activity as it related to small temperature changes showed that the greatest activity was obtained when the enzyme preparations were incubated at 37°C. At this temperature, incubation of the flasks for longer than 15 min did not significantly increase the amount of ocnjugation.

The type of buffer used for incubating the enzyme with 1-naphthol proved to be important in maximizing the enzyme activity. The pH of the buffer was less critical. With all buffers, the optimum pH was 7.0 but the Tris-HCl buffer increased the enzyme activity about 20 and 30% over that of the phosphate and Tris-maleate buffers, respectively.

The most important single factor in the glucuronidation of 1-naphthol was the presence of the correct cofactor. With the microsomal fraction of the liver, the conjugation of the substrate occurred only when the cofactor was UDPGA. Even the very similar compound, UDPG, did not elicit a trace of 1-naphthol conjugation by the microsomes. The intestine enzyme, being a 12,000g soluble fraction, probably contained some endogenous UDPGA since a small amount of conjugation of the substrate was noted when cofactors other than UDPGA were used. When these other cofactors were used, however, the degree of conjugation was increased by adding a greater quantity of cofactor. This was unlike the response to UDPGA where an increased concentration cuased in increase in the amount of substrate conjugated. To maintain the amount of 1-naphthol conjugation between 75 and 85% during routine assays, a standard concentration of 1.4 umoles of UDPGA per incubation were used. Mixing various cofactors with UDPGA did not enhance the enzyme activity but, in fact, caused a decrease when the added cofactor was either $NADPH_2$, UTP, or ATP.

With all other conditions as stated previously, the percentage conjugation was 80 to 85% when the 1-naphthol concentration was 2.5×10^{-4} M. Addition of greater amounts of substrate gave smaller percentage conjugation as expected. The data suggested that substrate inhibition may occur at high 1-naphthol concentrations with the liver microsomal preparation, while enzyme saturation alone was observed with the intestine enzymes. The importance of using larger amounts of substrate is readily apparent when one considers this in vitro enzyme system as a means of synthesizing certain glucuronides in sufficient quantity for chemical and toxicological evaluations.

A series of metal ions was tested for their effect on the conjugating activing of the liver microsome and intestine enzymes. Without the addition of any metal ion, and with all other parameters equal, only 20% of the 1-naphthol-^{14}C was conjugated. Magnesium at 1.3×10^{-4}M concentration enhanced the amount of 1-naphthol conjugated by about 20%. With all other metals, there was a marked inhibition of enzyme activity. However, the degree of inhibition of the enzyme varied according to the enzyme source and metal involved. The greatest differences were noted with cobalt and sodium where these metals inhibited the intestine enzyme more than the enzyme from the microsomes. The other metals inhibited the microsome enzyme activity to a greater degree.

Since magnesium was the only metal which enhanced conjugation, its concentration for maximum enzyme activity was determined. The addition of up to 2.5×10^{-4}M concentration of magnesium, 100 umoles of magnesium chloride per flask, enhanced the activity of the enzymes from the microsomes. This level of magnesium inhibited the intestine enzyme by about 10% its maximum activity.

Stability of the stored enzymes - Both enzyme sources were stored at -20°C as buffer solutions containing 250 mg tissue-equivalents per milliliter. The rat liver microsomal enzyme was more stable under these conditions than enzyme from the intestine; half-lives of the enzymes under storage conditions were 25 days and 2 days, respectively.

This difference in stability during storage may suggest that the conjugating enzymes in the microsomes were different from those in the intestine. However, the intestine enzyme preparation was only slightly more than a crude homogenate and may have contained certain components which denatured the enzyme at a rather rapid rate.

Inhibition of glucuronidation - The scientific basis for establishing the mode of action of insecticide synergists considered only those techniques which measured their effect on oxidative metabolism (Casida, 1970). This situation prompted an evaluation of their effect on the rat liver glucuronidation system reported herein. All of the synergists inhibited the conjugation of 1-naphthol by the liver microsomes and intestine enzymes (Table 58). However, a greater concentration was required to inhibit 50% of the microsomal enzymes. These data demonstrate that certain insecticide synergists do inhibit glucuronidation in an in vitro system; although, the significance of this inhibition, insofar as the effectiveness of insecticide synergists is concerned, can not be fully evaluated at this time. A series of insecticides including DDT, parathion, carbaryl, and rotenone did not inhibit glucuronidation at the 10^{-3}M concentration.

Based on the data presented in Table 59, it would be justified to suggest that the inhibition of conjugation does contribute to the action of insecticide synergists. These data show that while certain of the synergists, MGK-264 and piperonyl butoxide, are much greater inhibitors of oxidative metabolism than conjugative metabolism, other materials are approximately equal in their effectiveness. For example, the I_{50} of Tropital against the glucuronyl transferase of the microsomes was 1.0×10^{-3}M, but was 8.0×10^{-3}M against the microsomal enzymes which oxidized aldrin to dieldrin. These differences in the degree of effectiveness of the insecticide synergists in inhibiting metabolic processes offer encouragement to our quest for compounds which are better and more selective inhibitors of conjugation than those evaluated thus far.

Effect of controlled glucuronidation on the in vitro metabolism of carbaryl - Whether the inhibition of conjugative mechanisms of metabolism have any practical significance in the action of insecticides depends upon the effect of conjugation on the normal detoxication of the parent insecticide and/or of its toxic metabolites. This facet of glucuronidation was tested indirectly using carbaryl, a carbamate insecticide which is conjugatively metabolized by most organisms.

First, it was established that carbaryl-naphthyl-^{14}C was not metabolized by rat liver enzymes unless the cofactor $NADPH_2$ was added to the system, even though UDPGA was at its standard concentration of 1.4 umoles per incubation (Table 60, control sample). With no UDPGA added, but with 2 umoles of $NADPH_2$, 17% of the carbaryl was metabolized to organoextractable products or nonconjugates, and 9% to conjugated materials. This latter enzyme preparation was typical of the type of in vitro system used for the microsomal oxidation of insecticides.

Controlling the degree of glucuronidation by limiting the quantity of UDPGA in the system gave some insight into the effect of inhibition of conjugation on the metabolism of carbaryl. At the 5-umole level of UDPGA, 50% of the carbamate was conjugatively metabolized. As the cofactor level decreased, there was a corresponding decrease in conjugation, a corresponding increase in the amount of unmetabolized carbaryl, and no noticeable difference in the quantity of organoextractable metabolites produced (Table 60). Although carbaryl was metabolized by the microsomal enzymes to the extent of about 25% even when UDPGA was not added (Table 60), washing the microsomes reduced the metabolism of the carbamate to less than 10%, apparently by reducing the quantity of endogenous UDPGA. The failure of the organoextractable products to accumulate was somewhat surprising because they are composed of free hydrolytic and oxidative metabolites of carbaryl. Since the $NADPH_2$ concentration, and all other conditions except the UDPGA levels, were kept constant and were suitable for the continuation of oxidative metabolism, an accumulation of the oxidative metabolites would have been a likely response to decreased conjugation. That this is not the case, combined with the decreased total metabolism of carbaryl, suggest that oxidative and hydrolytic metabolism of the carbamate cannot continue unless the intermediate metabolites are converted to conjugated products. These data support the type of action reported for certain monoamine oxidase inhibitors on the metabolism of carbaryl in rats.

The substrate specificity of the microsomal conjugating system using a selected number of carbaryl metabolites was determined. Naphthol and 1-naphthyl N-hydroxymethylcarbamate were conjugated at about the 90% level while 40% of the 5-hydroxy- and 6-hydroxy derivatives of carbaryl were conjugated by the same enzyme preparation. The 5,6-dihydro-5,6-dihydroxy-1-naphthyl N-methylcarbamate did not form a glucuronide. According to Sullivan et al. (1972), the glucuronide of this product was a major metabolite in the urine of rats treated with carbaryl. This being the case, it was unclear as to why the in vitro glucuronidation of 5,6-dihydro-5,6-dihydroxycarbaryl was unsuccessful.

Carbaryl, per se, was not conjugated by the microsomal glucuronidation system. As pointed out earlier, the cofactor $NADPH_2$ was necessary for this material to be metabolized in any manner by the microsomes. The same was true for the compound Banol, the N-methylcarbamate of 6-chloro-3, 4-dimethylphenol. Although Baron and Doherty (1967) reported that as much as 18% of an oral dose of carbonyl-^{14}C-Banol to rats was excreted in the urine as a metabolite thought to be Banol-N-glucuronide, this product was not formed by the rat liver glucuronidation system. Glucuronide conjugates were formed by the microsomes only when $NADPH_2$ was added to the incubation mixtures.

Glucuronidation is but one of many forms of conjugative mechanisms which operate in the animal body. However, it is a very important mechanism, and one that is very much involved in the metabolism of insecticides. The current study provides a basis for considering glucuronidation, and maybe conjugation in general, as a means by which the toxic action of chemicals in the body might be controlled. For example, increasing conjugative activity through the use of certain chemicals could cause a more effective detoxication and elimination of the toxicant from the body. In essence, such chemicals would act as antidotes. Contrarily, compounds which would inhibit conjugative metabolism would likely enhance the action of a toxicant or drug, an effect which could be either disastrous or advantageous.

An intriguing possibility which could arise from an intensive investigation of the comparative conjugation mechanisms in insects and higher animals is the phenomenon of selective toxicity. Because glucuronides are formed by mammals while glucosides are formed by insects, there is a basic biochemical difference in the conjugative mechanisms of the two animal groups. Thus, a means of inhibiting insect glucosylation without inhibiting mammalian glucuronidation could serve to make a toxicant exhibit selective toxicity toward the insect species.

TABLE 57. IMPORTANCE OF CONJUGATION IN THE METABOLISM OF CARBAMATE INSECTICIDES IN RATS

Compound	Percentage of dose conjugated[a]
Banol	88.8
Carbaryl	82.6
Carbofuran	91.0
Formetanate	67.2
Meobal	85.0
Mobam	78.0

[a] Percentage of radioactive dose excreted as water-woluble metabolites in the urine by 48 hr after treatment.

TABLE 58. EFFECT OF CERTAIN INSECTICIDE SYNERGISTS ON CONJUGATION OF 1-NAPHTHOL BY RAT LIVER MICROSOMES AND INTESTINE ENZYMES[a]

	Molar concentration for 50% inhibition	
Compound[b]	Microsomes	Intestines
Isosafrole	10.0×10^{-3}	4.7×10^{-3}
MGK-264	3.0×10^{-3}	1.0×10^{-3}
Piperonyl butoxide	6.0×10^{-3}	1.7×10^{-3}
n-Propyl isome	7.0×10^{-3}	2.0×10^{-3}
Safrole	9.0×10^{-3}	4.2×10^{-3}
Sesamex	5.9×10^{-3}	1.9×10^{-3}
Sulfoxide	0.3×10^{-3}	1.2×10^{-3}
Tropital	1.0×10^{-3}	0.8×10^{-3}

[a] Conditions for conjugation described previously.

[b] The I_{50} values for the following compounds exceeded 10^{-2}M concentration: RO-5-1557, RO-5-1923, and RO-7-0165.

TABLE 59. INHIBITION OF RAT LIVER MICROSOMAL EPOXIDATION OF ALDRIN BY CERTAIN INSECTICIDE SYNERGISTS

Inhibitor	I_{50}[a]
MGK-264	0.1×10^{-3}
Piperonyl butoxide	0.1×10^{-3}
Sulfoxide	0.5×10^{-3}
Sesamex	1.0×10^{-3}
Tropital	8.0×10^{-3}

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[a] Conditions same as described previously except that 2 umoles of $NADPH_2$ was substituted for UDPGA and 20 ug aldrin for 1 umole of 1-naphthol.

TABLE 60. THE EFFECT OF REDUCED GLUCURONIDATION ON THE TOTAL METABOLISM OF CARBARYL-NAPHTHYL-^{14}C BY RAT LIVER MICROSOMES WITH $NADPH_2$[a]

	Percentage of radioactivity recovered as		
umoles UDPGA	Carbaryl	Organo-extractable metabolites	Glucuronide conjugates
Control[b]	88.8	0.0	0.0
5	23.4	15.4	49.8
3	28.9	18.3	42.0
1	34.3	16.3	36.9
0.3	48.9	17.5	22.8
0	63.9	16.8	9.2

[a] Carbaryl-^{14}C, 30 ug, incubated for 2 hr under same conditions described previously, except that 2 umoles of $NADPH_2$ were added to all flasks but the control.

[b] No $NADPH_2$ added but containing 1.4 umoles of UDPGA.

Mechanisms in Insects

Carbamate insecticides are conjugatively metabolized in insects with the major conjugates existing as glucosides, sulphates, and phosphates (Kuhr, 1970). Although individual glucosides have not been quantitated or identified, it is commonly suspected that glucoside formation is an important conjugation mechanism in insects.

Much of the literature in the area of insecticide metabolism deals with the initial oxidative modification of insecticidal chemicals. Although many investigators have reported the formation of a variety of glucoside conjugates, the mechanisms of their formation, their toxicological significance, and their relationship to important phenomena such as synergism, insecticide resistance, and the selective nature of insecticides have not been investigated.

Because conjugation is a major pathway for toxicants and this pathway is sensitive to certain chemicals, a detailed study was undertaken to develop and standardize an in vitro system for glucosylation reactions in insects. Tobacco hornworms (*Manduca sexta* Johan) were routinely used for insect tissue preparations. An easily conjugated material, 1-naphthol, was selected as a suitable substrate for studying the enzyme responsible for glucosylation in insects.

Methods and Materials

Enzyme assay - Early fifth instar, actively feeding tobacco hornworms were obtained from a culture maintained on an artificial diet. The worms were bled by cutting the horn and the blood collected. Fat body and fore-, mid-, and hindgut tissues were collected from the dorsally opened worms. The gut contents were removed and all tissues were homogenized in 5 vol. of Tris-HCl (0.05 M, pH 8.5) buffer. Crude homogenates were used to assay the UDP-glucosyl transferase activity.

Later, low centrifugation supernatants and other centrifugal fractions were tested for activity. When houseflies were used, whole insects were homogenized and utilized as whole body homogenates or fractionated by centrifugation and then the various fractions assayed for enzyme activity.

The reactions were carried out in 25 ml Erlenmeyer flasks. Naphthol-1-^{14}C was introduced into the flasks in 0.2 ml of chloroform and the solvent removed by a gentle jet of air, leaving a uniform layer of substrate in the bottom of the flask. Other constituents of the reaction mixture were added to the flask as buffer solutions. After adding the co-factor, the flasks were transferred to a metabolic shaker with the water temperature adjusted to the desired level. The reactions were shaken for the desired length of time and the reactions terminated by the addition of 1 ml of diethyl ether.

Extraction and radioassay - Extraction of unreacted 1-naphthol-1-^{14}C was accomplished by transferring the reaction mixture into 15 ml glass-stoppered centrifuge tubes. The reaction mixtures were extracted twice with 2 vol. of ether, which gave a recovery of free 1-naphthol of 95 percent. After combining the ether extracts and drying them with anhydrous sodium sulphate, the volume was adjusted to 10 ml and 1 ml aliquots were radioassayed by liquid scintillation counting. An aliquot of 0.2 ml of the aqueous layer was radioassayed to determine the water soluble naphthyl glucoside formed.

Ether extracts were concentrated and chromatographed on Chromar 500 sheets and developed two-dimensionally in various combinations of the following solvent systems: 1:5 ether-hexane; 2:1 methylene chloride-ethyl acetate; 4:1 ether-hexane. The aqueous phase was concentrated and subjected to beta-glucosidase for 24 hr (Kuhr and Casida, 1967) and the reaction mixtures extracted twice with 2 vol. of ether. The two solvent phases were radioassayed and the ether layer examined on tlc to determine the nature of the aglycone.

Effect of various chemicals on glucosylation- The chemical being tested for its effect on the conjugating enzyme was introduced into a reaction flask as an ether or buffer solution, just after the substrate was added. When ether was used, the solvent was evaporated by using a gentle jet of air. Other constituents of the reaction mixture were added in the manner described earlier and then the reactions carried to completion. Changes in the amount of conjugation of 1-naphthol were determined and the degree of reduction of 1-naphthol glucoside formation was expressed as percentage inhibition as compared to that of control sample.

Epoxidation of aldrin by tobacco hornworm enzymes - To determine the relative effectiveness of certain insecticide synergists on oxidative and conjugative metabolism, a limited number of experiments was conducted on the epoxidation of aldrin by hornworm midgut enzyme. A typical reaction mixture contained 20 ug of aldrin in 100 ul of methylcellusolve; 2 umoles of $NADPH_2$ in 0.5 ml of buffer; 2.9 ml of Tris-HCl (0.05M) buffer, pH 8.5; 0.5 ml of tobacco hornworm midgut, 5000g supernatent (200 mg tissue equivalents), and 0.1 ml of ethanol containing the desired amount of the inhibitor. The mixtures were incubated for 1 hr at 37°C in a metabolic shaker. At the end of the incubation period, the reactions were terminated by adding 4 ml of 2:3 mixture of isopropanol-hexane and extracted twice with 2 vol. of hexane.

The organic solvent phase was analysed for aldrin and dieldrin by gas chromatographic means using a Varian Aerograph Model 1700 instrument equipped with an electron capture detector. Operating parameters were as follows: column: glass, 6 ft x 1/8 in. i.d., packed with 10% DC 200 on Anachrom ABS, 80-90 mesh; carrier gas: nitrogen, 45 ml/min; temperature: column 195°C, injection port 200°C, detector 215°C. Retention times for aldrin and dieldrin were 3.8 and 9.6 min, respectively.

Results and Discussion

Parameters for conjugation - The conditions necessary to obtain maximum conversion of 1-naphthol-^{14}C to 1-naphthyl glucoside by tobacco hornworm midgut enzymes are shown in Table 61. Under these conditions about 60 percent of the 1-naphthol was conjugated, yielding a material which could not be extracted from the aqueous reaction mixture with ether. Tlc analysis of the radioactivity extracted into the ether demonstrated that over 95% was the starting material. Occasionally, 2 minor components other than 1-naphthol were observed. However, they were not present in quantities sufficient for identification. It is possible that they were diol derivatives of 1-naphthol.

Treatment of the water-soluble material with beta-glucosidase or with 2N HCl yielded a single ether extractable product identified as 1-naphthol. Total recovery of the radioactivity originally in the water layer exceeded 92 percent. These data showed conclusively that 1-naphthol was conjugated directly with glucose supplied by the cofactor UDPG without undergoing any other type of chemical modification. Because of the extraction characteristics of the conjugate and aglycone, simple extraction of the enzyme reaction mixture with ether, and subsequent radioassay of the two phases, were all that was required to determine the amount of substrate conjugated. The optimum conditions for conjugation shown in Table 61 were established using this technique.

The correct cofactor proved the most critical component in the in vitro system used to conjugate 1-naphthol by the hornworm midgut enzyme. Of 6 different cofactors tested, only UDPG supported the conjugation of the substrate. Minute quantities of conjugation observed with the other substrates were attributed to the presence of endogenous UDPG. This was supported by the fact that only with UDPG did an increased amount of cofactor, cause a corresponding increase in conjugate formation.

Although not as critical as the cofactor, the substrate concentration was an important factor in its conjugation. Maximum production of naphthyl glucoside occurred when the 1-naphthol concentration was 2.5×10^{-4}M. At levels above this concentration, the rate of glucoside formation was lessened considerably.

Enhancement of the midgut enzyme activity by the addition of metal ions occurred only if the metal was magnesium. Several metals, particularly cadmium, mercury, and zinc, were potent inhibitors of the conjugating enzymes when their concentration in the final incubation mixture was as low as 1.3×10^{-5}M. Increasing the concentration of magnesium up to 2.5×10^{-2}M resulted in a linear increase in the production of naphthyl glucoside. However, higher amounts of the metal inhibited the conjugation reaction. Of the four buffers used in the study, Tris-HCl supported maximum glucoside formation. Glycine-sodium hydroxide was almost as effective, while phosphate and Tris-maleate were the poorest. With all buffers, the optimum pH was 8.5. Hence, Tris-HCl, pH 8.5 (0.05M), buffer was used for all standard incubation.

Reacting the enzyme preparations at temperatures from 20 to 79°C demonstrated that best results were obtained at 37°C. Glucoside formation was reduced at temperatures less than 30°C or higher than 50°C. Incubations carried out for varied lengths of time showed that over 95 percent of the total naphthyl glucoside was formed after only 15 min of incubation. Therefore, all standard incubations were made at 37°C for 15 min in a metabolic shaker.

Stability of the enzyme - Enzyme activities of the hornworm midgut enzyme and fat body homogenate were compared during 50 days of storage. The enzyme preparations were stored at -20°C as buffer solutions and thawed immediately prior to use. Although fresh fat body preparations were more active than the midgut, this activity dropped precipitously upon storage. After the initial drop in activity from the fat body, both the enzyme sources lost activity at a comparable rate. When blood, fat body and foregut and hindgut tissues from tobacco hornworms were compared for naphthyl glucosylation, the fat body was more active as calculated on the basis of the fresh weight of the tissue. However, since this activity was reduced considerably by storage and centrifugation, the midgut was used in this study as the major enzyme source.

To determine the centrifugal fraction containing the greatest enzyme activity, homogenates of both tobacco hornworms and adult houseflies were assayed for naphthyl glucosylation activity (Table 62). The whole body homogenates and the 15,000g supernatant fraction of the hornworms were equally active while the latter fraction from the houseflies was less active than the crude homogenate. Further centrifugation showed that most of the activity was located in the 15,000g pellet of the houseflies. The activity in the hornworm was contained in the 105,000g soluble fraction. That enzyme activity in the 15,000g supernatant from flies was associated entirely with the microsomal pellet following centrifugation at 105,000g.

Effect of synergists - A number of synergists were tested for their effect on naphthyl glucoside formation by tobacco hornworm enzyme (Table 63). Sulphoxide was the most active of the compounds tested. Tropital, MGK-264, and piperonyl butoxide were other compounds found to be inhibitory at the 10^{-3}M concentration, while Sesamex was a poor inhibitor of conjugation. Other compounds tested (inosafrole, n-propyl isome, RO-5-1923, RO-7-0165, and RO-5-1557) were required in excess of 10^{-2}M concentration for 50 percent inhibition of naphthyl glucosylation.

Comparison of the ability of these synergists to inhibit a conjugative and an oxidative reaction was made using the tobacco hornworm midgut enzyme. Aldrin epoxidation was selected as the oxidation reaction. As evident by the data in Table 63, all the synergists tested were much better inhibitors of aldrin epoxidation than naphthyl glucosylation.

Sulphoxide and piperonyl butoxide were the most effective inhibitors of aldrin epoxidation.

This preliminary investigation into glucosylation in insects had established parameters for studying this important metabolic mechanism. Additionally, it demonstrated that the glucosyl transferase enzyme is sensitive to a number of compounds recognized as insecticide synergists. Although these particular synergists were more potent inhibitors of aldrin epoxidation, there may be other compounds which would more selectively inhibit the conjugation reactions. It is now possible to more accurately evaluate the nature of the glucosyl transferase in insects and to attempt to utilize this system for more desirable methods of insect pest control.

TABLE 61. COMPOSITION OF THE REACTION MIXTURE USED FOR THE IN VITRO CONJUGATION OF 1-NAPHTHOL BY TOBACCO HORNWORM MIDGUT ENZYME

Substrate	1-Naphthol-1-^{14}C, 0.1 umole (2.5 x 10^{-5}M)*
Buffer	Tris-HCl, 0.05M, 8.5 pH, 1.0 ml
Metal	$MgCl_2$, 100 umoles in 1.0 ml buffer (2.5 x 10^{-2}M)*
Cofactor	UDPG 1.5 umole in 1.0 ml buffer (3.8 x 10^{-4}M)*
Enzyme	100 mg equivalent tissue in 1.0 ml buffer
Incubation	37°C for 15 min, with shaking

* Molar concentration in final incubation mixture of 4 ml.

TABLE 62. 1-NAPHTHOL GLUCOSYLATION ACTIVITY OF WHOLE BODY EXTRACTS FROM ADULT HOUSEFLIES AND TOBACCO HORNWORM LARVA

	Conjugation of 1-naphthol (%)	
Fraction	Tobacco hornworm	Housefly
Crude homogenate	50.5	48.8
15,000g Supernatant	51.2	21.1
15,000g Pellet	7.3	45.8
105,000g Supernatant	39.9	6.0
105,000g Pellet	5.8	24.3

TABLE 63. SENSITIVITY OF 1-NAPHTHOL GLUCOSYLATION AND ALDRIN EPOXIDATION ENZYMES FROM TOBACCO HORNWORM MIDGUT TO SOME INSECTICIDE SYNERGISTS

	Molar concentration for 50% inhibition of	
Compound	1-Naphthol conjugation	Aldrin epoxidation[a]
MGK-264	5.5×10^{-3}	1.0×10^{-5}
Piperonyl butoxide	6.3×10^{-3}	4.4×10^{-6}
Sesamex	1.0×10^{-2}	7.5×10^{-5}
Sulphoxide	1.2×10^{-3}	8.8×10^{-6}
Tropital	5.3×10^{-3}	3.0×10^{-5}

[a] Same as in Table 61 except that UDPG was replaced with 2 umoles of $NADPH_2$ and 1-naphthol was substitured by 20 ug of aldrin and incubated for 1 hr.

Factors Influencing Conjugation

Having established that conjugation is a major metabolic pathway for carbamates in animals, a study was undertaken to further characterize the conjugative enzymes in various mammals and insect species using a well defined in vitro conjugating system. Also, the effect of age, sex, resistance and toxicity of other chemicals on conjugation was evaluated.

Methods and Materials

Animals - Mammalian livers were obtained from St. Louis Serum Company, 114-120 St. Claire Ave., East St. Louis, Illinois or removed from rats maintained in the laboratory. The multi-resistant and susceptible strains of the housefly were obtained from the United States Department of Agriculture, Entomology Research Division, Gainesville, Florida.

In vivo metabolism of 1-naphthol by houseflies - Adult houseflies, 5 days old, were anesthized with carbon dioxide and then treated topically with 1-naphthol-1-^{14}C at a rate of 0.022 ug/per fly. This amounted to about 5,085 dpm per insect and was adequate for evaluating conjugation since 100 flies were processed as a single replicate. A detailed examination of 1-naphthol conjugates was conducted 4 hours after treatment of the flies.

Radioactivity in the flask (excreta) plus the radioactivity remaining on the surface of the flies was removed by rinsing the flask and insects several times with water and then with ether. Free 1-naphthol in the ether layer was determined and the 1-naphthol conjugates in the water was evaporated to dryness, dissolved in a small amount of methanol, and spotted on tlc. Internal radioactivity was extracted by homogenizing the treated flies in a 1:1 acetone-water mixture. The filtered extract was analyzed in the manner described for the excreta.

In vitro glucosylation of 1-naphthol - Various insect species were used as an enzyme source to compare their ability to conduct glucosylation reactions. The standard enzyme for glucosylation was prepared by homogenizing the whole insect in 0.05 M Tris-HCl buffer, pH 8, to yield a 20% (w/v) homogenate. The homogenate was filtered through two layers of cheesecloth to remove gross debris and the filtrate homogenized in a Potter Elvehyem type cell homogenizer. The crude homogenate was centrifuged at 5,000g for 10 minutes using a Beckman Model L2-65 untracentrifuge and, the supernatant was used as the enzyme source.

The conjugative abilities of different insect tissues also were examined. Early fifth instar, actively feeding tobacco hornworm larvae were bled by cutting the horn and the blood collected. Fat body, midgut, and malphigian tubules were collected from the dorsally opened worms. The gut contents were removed and all tissues were homogenized in 5 volumes of Tris-HCl (0.05 M, pH 8) buffer, centrifuged, and the 5,000g supernatant assayed for enzyme activity.

The distribution of glucosyltransferase in subcellular fractions of housefly whole-body homogenates was examined by separating the homogenates into various centrifugal fractions. The first fraction assayed was the crude homogenates, while the second and third fractions consisted of the supernatant of the crude homogenates after centrifuging at 5,000g for 10 minutes. The precipitates after dispersal in a volume of Tris-HCl buffer that made the suspension equivalent to the original 20% homogenate were assayed also. The next fractions were prepared by centrifuging the crude homogenates at 15,000g for 30 minutes and analyzing both the supernatant (microsomes + solubles) and the precipitate (mitochondria). Two other fractions were prepared by centrifuging the 15,000g supernatant at 105,000g for 1 hour. The supernatant obtained after centrifugation at 105,000g was used as an enzyme source, as was the particulate fraction (microsomes). All procedures were conducted at 0-4°C to avoid loss of enzyme activity.

A typical incubation mixture contained 1.3 x 10^{-3} micromoles of radiolabeled 1-naphthol (44,000 dpm per incubation); 100 mg equivalents of insect tissues in 0.5 ml buffer; 2.9 ml of 0.5 M Tris-HCl buffer (pH 8); 0.1 ml (2 mg) of $MgCl_2$; and 0.5 ml (1 mg) of UDPG. The molar concentrations for $MgCl_2$ and UDPG in the final 4 ml incubation volume were 2.5×10^{-3} M and 4.12×10^{-4}, respectively.

The 1-naphthol, in organic solvent, was transferred to a 25 ml Erlenmeyer glask, and the solvent evaporated with care to deposit 1-naphthol uniformly over the bottom of the flask. The other constituents were added and the flasks were transferred to a metabolic shaker with the water temperature at 37°C. The cofactor was added and the incubation time, 15 minutes, was recorded from that point. Reactions were terminated by the addition of 2.0 ml of diethyl ether to the flask.

Unreacted 1-naphthol-1-^{14}C from the enzyme preparations was extracted by transferring the reaction mixture to a 15 ml glass-stoppered centrifuge tube and extracting once with 10 ml of diethyl ether. Further extraction was not required since over 95% of the free 1-naphthol was recovered. Volumes of both the ether and the water layers were recorded and aliquots, 1 ml ether and 0.2 ml water, were radioassayed.

In vitro glucuronide conjugation of 1-naphthol - Animal tissues were weighed and sufficient Tris-HCl buffer (pH 7.0) to yield a 25% (w/v) tissue homogenate was added, then the tissue was cut into small pieces and homogenized in a Virtis homogenizer for 4 minutes. The homogenate was further homogenized until the cells were completely broken.

Various subcellular fractions were isolated from the homogenate by differential centrifugation. The crude homogenate was first centrifuged at 15,000g for 30 minutes and the supernatant decanted into clean centrifuge tubes and spun at 105,000g for 60 minutes. The microsomal pellet was homogenized in Tris-HCl buffer of sufficient volume to achieve reconstitution to the original 25% homogenate. Therefore, 1 ml of the microsomal suspension contained the microsomes from 250 mg of tissue. All the above operations were carried out at 0-4°C.

For incubation, 125 mg equivalents of rat tissue in 0.5 ml buffer; 2.9 ml of 0.05 M Tris-HCl buffer (pH 7); 0.1 ml (2 mg) of $MgCl_2$; and 0.5 ml (1 mg) of UDPGA were added to a 25 ml Erlenmeyer flask containing 1.3×10^{-3} micromoles of the labeled 1-naphthol. The molar concentrations for $MgCl_2$ and UDPGA in a final incubation volume were 2.5×10^{-3}M and 3.9×10^{-4}M respectively. The preparations were incubated at 37°C for 15 minutes.

Metabolite identification - The conversion of 1-naphthol to water soluble products was used as a general indication of the conjugative enzyme activity in both in vivo and in vitro studies. Therefore, the water phase of the enzyme preparations and of the extract of houseflies and excreta were radioassayed after extraction with organic solvent. The percentage conjugation of the applied 1-naphthol was calculated from these data.

From more critical evaluation of the nature of the water soluble products, these materials were analyzed by tlc. Approximately 50 ug of 1-naphthol glucoside, 1-naphthol sulfate and 1-naphthol standards were added to the water soluble conjugates which were dissolved in methanol. The mixture was applied to Chromar tlc sheets and developed first in hexane; this step did not move any radioactivity from the origin but did remove fats and other interferring materials. The tlc was then developed in a 5:1 mixture of chloroform and methanol containing 10% acetic acid. A 25% aqueous solution of sulfuric acid was sprayed on the plates to detect the nonlabeled standards, and the radioactive areas were located by radioautography. The radioactive conjugates were extracted from the tlc plates and quantitated by liquid scintillation counting.

Stability of the enzymes during storage - Glucosyltransferase activity in the housefly and cockroach were assayed after storage at various temperatures. The form of storage was either as the whole insects at -20°C after which the 5,000g supernatant was prepared, or the 5,000g supernatant stored at -20°, 0°, and 25°C. The frozen samples were stored as small individual preparations to avoid loss of activity during thawing and refreezing. For each analysis 100 mg equivalent of tissue were used.

The rat liver, kidney and intestine were stored at -20°, 0° and 25°C either as whole tissues in Tris-HCl buffer and the microsomal enzymes then prepared, or the microsomal pellet was prepared from livers of freshly killed rats and stored. Individual, rather than pooled samples were stored to avoid loss of activity during thawing and refreezing. The effect of storage at -20°C on the 15,000g supernatant enzyme preparation of rat kidney, lung, small intestine, caceum, large intestine, heart, stomach, fat and brain also was determined.

Oxidative metabolism - The method used by Hart and Fouts (1965) to measure the rate of TPNH oxidation by the rat liver microsomal enzymes was employed. The cuvet used as a blank contained 40 mg equivalents of tissue in a total volume of 3 ml of 0.05 M Tris-HCl buffer, pH 7.4. The cuvet used for oxidase assay contained 0.36 umole TPNH, and 40 mg equivalents of tissue in a total volume of 3 ml of 0.05 M of Tris-HCl. The optical density was measured at 340 mu in a model DV Beckman spectrophotometer.

Sensitivity of glucuronyltransferase to various insecticides - DDT, carbaryl, parathion, tedion, dipterex and disulfoton were evaluated for their effects on rat liver microsomal glucuronyltransferase activity. The insecticides were introduced into a reaction flask as an acetone solution just after the substrate was added. The acetone was evaporated using a gentle jet of air and other constituents of the reaction mixture were added and the reactions carried to completion. Sufficient quantities of each insecticide were added so that their concentrations in the incubation mixtures were 10^{-5}, 10^{-4} and 10^{-3} molar.

Results and Discussion

Stability of glucosyltransferase during storage - The effects of storage on glucosyltransferase activity in the housefly showed that the enzymes stored as a 5,000g supernatant at -20° and 0°C were stable throughout 10 days storage period. When the enzyme was stored at 25°C, the glucosyltransferase enzymes retained 90% of its initial activity after 24 hrs but then the activity declined rapidly. The preparation from cockroaches which were allowed to feed until the time the enzyme was prepared lost 53% of its activity in 4 days and 100% after 7 days, whereas in cockroaches not fed for 24 hrs before preparing the enzyme, there was only 12% loss after 4 days and 27% loss of activity after 7 days.

The stability of the housefly TPNH oxidase activity was examined using the 5,000g supernatant prepared from a whole body homogenate after the insects were stored for various periods of time. In this case, about 25% of the initial activity was lost after 10 days of storage. However, an increase in the enzyme activity, a maximum of 45% during the first 24 hrs of storage was observed.

In vitro glucosylation by different insect species - The enzyme activity was assayed using a 5,000g supernatant of tobacco hornworm larvae, Manduca sexta (Lepidoptera), housefly, Musca domestica (Diptera), Indian meal moth, Plodia interpunctella (Lepidoptera), alfalfa weevil, Hypera postica (Cleoptera) and American cockroach, Periplaneta americana (Orthoptera). The results outlined in Table 64 indicate that: - (1) Each of these insects were capable of conjugating about 77% of 1-naphthol to form 1-naphthol glucoside. No other metabolites were detected. (2) The 5,000g supernatant of the head, thorax and abdomen of houseflies were individually as effective in conjugating 1-naphthol as the whole body homogenate. (3) The conjugative activity of various tissue preparations from the tobacco hornworm larvae was similar to that of a whole body homogenate.

Examination of glucosyltransferase activity in subcellular fractions of housefly homogenates showed that crude homogenates of the whole body, the 5,000g and 15,000g supernatants as well as the 5,000g, 15,000g and 105,000g precipitates were all equally active in conjugating 1-naphthol (Table 65). With these preparations, from 67 to 75% of the 1-naphthol was conjugated. The corresponding value for the 105,000g supernatant was 44.6%.

Influence of age, sex and insecticide resistance - The glucosyltransferase enzyme was assayed using the 5,000g supernatant prepared from the resistant and susceptible houseflies at various stages of development. Eggs of both strains were high in glucosyltransferase activity while the enzyme activity was lower in 3- and 5-day-old housefly larvae (Table 66). The level of glucosyltransferase activity in the pupal stage was comparable to that in the eggs and in the adult flies. Neither age, sex or insecticide resistance had any influence on the conjugation of 1-naphthol by the adult insects.

The fate of the radiocarbon after topical application of 1-naphthol-1-^{14}C to susceptible and resistant houseflies is shown in Table 67. The data show that both strains of flies excreted the 1-naphthol very rapidly, with 50 to 60% of the dose eliminated by 4 hours. Although the individual metabolites of 1-naphthol in the excreta and flies differed quantitatively, it does not appear that insecticide resistance vastly effected overall conjugative metabolism in the housefly. It was established that glucosylation is an important in vivo metabolic reaction as was indicated by the earlier in vitro studies. After 4 hours, for example, approximately 13% of the applied 1-naphthol had been converted to the glucoside derivative.

Conjugation by different animal species - The conjugative enzyme was assayed using liver microsomes of various animal species (Table 68). The results showed that the guinea pig, chicken and hamster livers were more efficient in conjugating 1-naphthol than other livers. With these animals, over 85% of the added 1-naphthol was conjugated. The enzymes from the other animals conjugated less of the 1-naphthol but all showed very active enzyme activity. The distribution of glucuronyltransferase in several rat tissues other than the liver was examined. Data in Table 69 show that the liver and kidney had the greatest glucuronyltransferase activity per unit weight. The lung 15,000g supernatant formed the glucuronide in quantities almost equal to those from the liver and kidney. The activity of the glucuronide transferase in the alimentary tract was less active than that in the liver, kidney and lungs.

The conjugative enzyme activity in the alimentary tract seemed to be dependent on the presence or absence of the mucosa in tissues used for enzyme preparation. For example, small intestine, caceum and large intestine, which have large amounts of mucosa, gave higher activity than those found in the stomach, which had less mucosa. Washing the inside of the intestine until most of the mucosa was removed, reduced the enzyme activity by about 20%. This indicated that most of the enzyme activity was located in the mucosa and not in the muscular wall.

Stability of the glucuronyltransferase enzymes during storage - The effects of storage on the conjugative enzyme activity in rat liver, kidney and intestine stored as whole tissues and as microsomal pellets showed that the enzyme activity of rat liver and kidney was very stable during storage at -20°C. None of the activity of the enzymes was lost after 14 days of storage, and less than 10% of the original enzyme activity was lost after 90 days of storage.

Enzymes from rat intestine were not so stable and, depended to a certain extent on the type of preparation used for storage. For example, the most stable preparation was the microsomal enzymes from the stored intact intestine, as only 10% of the enzyme activity was lost after 14 days of storage at -20°C. Almost no activity remained after 90 days. On the other hand, enzymes stored as microsomal pellets were considerably less stable, with 90% of the enzyme activity lost after 14 days of storage.

The stability of the glucuronyltransferase enzyme prepared as a 15,000g supernatant from lungs, caceum, large intestine were considerably less stable than that in the liver and kidney (Table 69) with 30 to 40% of the enzyme activity lost after 10 days of storage. The small intestine, heart, stomach, fat and brain enzyme activity dropped 10 to 30% of the initial activity after 5 days and to 5 to 20% after 10 days of storage at -20°C.

The stability of enzymes in a 15,000g supernatant of rat liver when stored at various temperatures showed that none of the enzyme activity was lost after a week of storage at -20°C, and that only about 5% of the activity was lost after 10 days of storage at 0°C. When the enzymes were stored at 25°C, 89% of the initial activity was retained after 12 hours, 50% after 24 hours and only 8% after 2 days.

Sensitivity of rat liver glucuronyltransferase enzymes to insecticides - Several common insecticides were evaluated for their effect on the glucuronide transferase enzyme using the rat liver microsomal enzyme. The results of these were entirely negative. None of the insecticides inhibited the conjugating enzymes regardless of the level of pesticide added to the system.

TABLE 64. GLUCOSYLATION OF 1-NAPHTHOL-1-^{14}C BY THE 5,000g SUPERNATANT OF DIFFERENT INSECTS AND INSECT TISSUES[a]

Enzyme preparation	Percent conjugation of 1-naphthol
Housefly	
Whole body	77.1
Head	77.3
Thorax	80.6
Abdomen	74.5
Tobacco Hornworm Larvae	
Whole body	79.4
Whole body, less midgut	78.1
Midgut	72.6
Malpigian tubules	79.5
Fat body	78.0
Haemolymph	5.3
American Cockroaches	
Whole body	75.9
Whole body, less midgut	77.9
Midgut	77.8
Indian Meal Moth	
Whole body	77.4
Alfalfa Weevil Larvae	
Whole body	76.1

[a] Enzyme from 100 mg of tissue was used for each analysis.

TABLE 65. GLUCOSYLATION OF 1-NAPHTHOL-1-^{14}C BY SUBCELLULAR FRACTIONS OF A HOUSEFLY HOMOGENATE[a]

Centrifugal fraction	Percent conjugation
Crude homogenate	68.7
5,000g supernatant	71.0
5,000g precipitate	66.7
15,000g supernatant	70.4
15,000g precipitate	72.0
105,000g supernatant	44.6
105,000g precipitate	74.6

[a] Enzyme from 100 mg of tissue was used for each analysis.

TABLE 66. THE EFFECT OF AGE AND SEX ON THE CONJUGATION OF 1-NAPHTHOL BY SUSCEPTIBLE AND RESISTANT HOUSEFLY HOMOGENATES, 5,000g SUPERNATANT[a]

Enzyme source	Percent conjugation			
	Susceptible flies		Resistant flies	
Eggs	77.4		77.7	
Larvae, 3 days old	61.3		71.8	
Larvae, 5 days old	41.6		58.3	
Pupae	78.1		79.2	
Adults	Female	Male	Female	Male
1 day old	77.9	81.6	78.0	76.6
3 days old	77.8	77.2	78.7	77.1
5 days old	74.0	79.2	77.5	77.7
7 days old	79.8	79.6	81.6	78.7
11 days old	77.9	78.4	78.8	76.6

[a] Enzyme from 100 mg of tissue was used for each analysis.

TABLE 67. FATE OF TOPICALLY APPLIED 1-NAPHTHOL-1-^{14}C IN SUSCEPTIBLE AND RESISTANT HOUSEFLIES AFTER 4 HOURS

	Percent of applied dose	
Metabolites	Susceptible	Resistant
Excreta		
1-naphthol	24.0	15.5
1-naphthylglucoside	7.4	10.7
Unknown I	5.8	10.1
Unknown II	10.0	3.7
Unknown III	6.2	4.6
Total	61.6	53.8
Houseflies		
1-naphthol	3.4	1.1
1-naphthylglucoside	5.7	2.6
Unknown I	1.4	2.4
Unextractables	5.2	6.9
Total	15.7	13.0
Total recovery	77.3	66.8

TABLE 68. CONJUGATION OF 1-NAPHTHOL BY THE MICROSOMAL FRACTIONS OF LIVERS FROM DIFFERENT ANIMALS[a]

Source of liver enzyme	Percent conjugation	umoles 1-naphthol conjugated/g tissue/ hr. x 10^{-2}
Guinea Pig	90.4	3.8
Hamster	85.7	3.6
Chicken	84.9	3.5
Rat	76.6	3.2
Sheep	76.9	3.2
Pig	78.8	3.3
Quail	74.5	3.1
Cow	73.4	3.0
Rabbit	70.4	2.9
Dog	62.4	2.6
Fetal Pig	57.4	2.4

[a] Enzyme from 125 mg of tissue was used for each analysis.

TABLE 69. COMPARATIVE GLUCURONYLTRANSFERASE ACTIVITY IN THE 15,000g SUPERNATANT OF VARIOUS RAT TISSUES AND EFFECT OF STORAGE AT -20°C ON THE ENZYME ACTIVITY[a]

Source of Enzyme	Percent conjugation of 1-naphthol	Percent of initial activity after storage for:	
	0 time	5 days	10 days
Kidney	87.6	97.5	97.7
Liver	84.4	97.9	94.9
Lung	80.5	69.2	61.1
Small intestine	70.1	14.6	13.4
Caceum	67.5	69.2	65.6
Large intestine	66.0	62.5	68.0
Heart	55.0	14.2	10.4
Stomach	54.5	18.4	8.1
Fat	56.0	33.9	26.1
Brain	54.5	23.3	18.2

[a] Enzyme from 125 mg of fresh tissue used for each analysis.

Synthesis of Glycosides

Chemical syntheses and acute toxicity of the beta-D-O-glucosides of carbaryl metabolites, 4-(N-methylcarbamoyl-oxy)-1-naphthyl-beta-D-glucopyranoside and 5-(N-methylcarbamoyloxy)-1-beta-D-glucopyranoside are reported. The preparation of their respective decarbamylated products, 4-hydroxy-1-naphthyl-beta-D-glucopyranoside and 5-hydroxy-1-naphthyl-beta-D-glucopyranoside are also reported. These syntheses can provide material for toxicological evaluation and will enable identification of the intact plant conjugates without resorting to hydrolysis of the glycones. The synthetic conjugates may serve as standards to aid in the determination of the sugar moiety of carbaryl plant conjugates, an important consideration which up to this time has been lacking.

Methods and Materials

Chemicals - 1,4-Naphthalenediol and 1,5-naphthalenediol were purchased from Eastman Kodak Company. 4- and 5-Hydroxycarbaryl were synethesized by the reaction of the corresponding naphthalenediol with methyl isocyanate (Knaak et al., 1965). Beta-D (+) Glucose pentaacetate was purchased from Sigma Chemical Company, and boron trifluoride ether complex (98%) was purchased from Matheson Coleman and Bell. Methyl tetra-O-acetyl-beta-D-glucopyranuronate was synthesized by the reaction of a mixture of glucuronolactone, sodium methoxide and methanol with acetic anhydride and perchloric acid (Bollenback et al., 1955). Anhydrous methanol was prepared by the distillation of pesticide quality methanol over magnesium turnings (Fieser, 1957). Benzene used in the syntheses was dried over sodium.

Chromatography - Thin-layer chromatography was used to follow the progress of all reactions, and to determine the purity of reaction products.

Silica gel F-254 precoated plates (0.25 mm thickness, Brinkman Instruments, Inc., Westbury, New York) developed in either benzene-ether (7:3) or petroleum ether-chloroform-ethanol (7:2:1) were used to separate 4- and 5-hydroxycarbaryl glucoside tetraacetates from their respective starting materials and other reaction products. The R_f values for the 4- and 5-hydroxycarbaryl glucoside tetraacetates were identical in the benzene-ether system ($R_f = 0.16$) and in the petroleum ether-chloroform-ethanol mixture ($R_f = 0.46$). Spots were detected by visualization under ultraviolet, and by spraying with 10% methanolic sulfuric acid with subsequent baking at 140° for three minutes.

The glucosides of 4- and 5-hydroxycarbaryl were chromatographed on aluminum oxide F-254, Type T, precoated plates (0.25 mm thickness, Brinkman Instruments, Inc., Westbury, New York) developed in chloroform-methanol-acetic acid (75:15:10). The R_f values for the 4- and 5-hydroxycarbaryl glucosides in this system were also identical; $R_f = 0.49$. However, upon spraying the chromatograms with 10% methanolic sulfuric acid and baking them at 140°, the 4-hydroxycarbaryl glucoside stained a dark gray. The glycosides of 4- and 5- hydroxycarbaryl isolated from bean plants had identical R_f values on silica gel plates developed in chloroform-methanol-water (65:25:4) (Mumma et al., 1971).

Instrumentation - The infrared data were obtained using KBr pellets with a Beckman IR5A infrared spectrophotometer. the NMR spectra were determined with a Varian Model T-60 spectrometer using tetramethylsilane as the internal standard. The mass spectral data were recorded with a Finnigan Series 1015C mass spectrometer at 70 ev. The high resolution mass spectral data were obtained on a Hitachi RMU-7 mass spectrometer. Specific rotations were determined with a Bendix ETL-NPL Automatic Polarimeter, Type 143A. Melting points were determined with an Electrothermal capillary melting point apparatus and are uncorrected. Elemental analyses were performed by Galbraith Laboratories, Inc., Knoxville, Tenn.

Syntheses

Preparation of 4-hydroxycarbaryl glucoside tetraacetate (I) and 5-hydroxycarbaryl glucoside tetraacetate (II)- (1). 4-(N-Methylcarbamoyloxy)-1-naphthyl-tetra-O-acetyl-beta-D-glucopyranoside (I). A mixture of 7.2 g (0.033 mol) of 4-hydroxycarbaryl, 11.7 g (0.03 mol) of beta-D (+) glucose pentaacetate, 0.38 ml (0.003 mol) of boron trifluoride ether complex (98%), and 350 ml of anhydrous benzene was refluxed with stirring for 2 hr. The dark red solution was cooled to 4° and the excess 4-hydroxycarbaryl was removed by filtration.

The cooled filtrate was deacidified with a cold solution of 0.5 N sodium hydroxide (2 x 75 ml). The organic layer was separated, washed with ice water (4 x 100 ml), dried over magnesium sulfate, and concentrated. The dark red syrupy residue was dissolved in 40 ml of hot ethanol. The solid which formed on standing was filtered to give 8.70 g of a mixture of (I) and glucose pentaacetate, mp 95-125°. The mixture was stirred for 2 hr with 400 ml of diethyl ether in order to extract the unreacted glucose pentaacetate. Filtration gave 3.14 g (19%) of (I), mp 165-169°. A single recrystallization from ethanol gave 2.94 g (18%) of (I) as tiny, off-white needles, mp 169-171°. (2). 5-(N-Methylcarbamoyloxy)-1-naphthyl-tetra-O-acetyl-beta-D-glucopyranoside (II). A mixture of 7.2 g (0.033 mol) of 5-hydroxycarbaryl, 11.7 g (0.03 mol) of beta-D (+) glucose pentaacetate, 0.38 ml (0.003 mol) of boron trifluoride ether complex (98%), and 350 ml of anhydrous benzene was refluxed with stirring for 1 hr. The dark red mixture was allowed to cool to room temperature and filtered to give 2.0 g (28%) of crude unreacted 5-hydroxycarbaryl, mp 155-165°. Recrystallizaiton from ethyl acetate/hexane gave 1.51 g (21%) of 5-hydroxycarbaryl, mp 166-168° [lit. mp 166-167° (Knaak et al., 1965)].

The filtrate from the reaction mixture was cooled, washed successively with a cold solution of 0.5 N sodium hydroxide (2 x 75 ml) and ice water (4 x 100 ml), then dried over magnesium sulfate, and concentrated. The yellow syrupy residue crystallized upon addition of 1 l. of diethyl ether. Filtration afforded 4.20 g of a colorless solid which was recrystallized from ethanol to give 3.84 g (23%) of (II) as tiny, colorless needles, mp 165-168°.

Evaporation of the diethyl ether filtrate gave 7.55 g (65%) of crude unreacted glucose pentaacetate, mp 115-122°C. Recrystallization from ethanol gave 5.60 g (48%) of glucose pentaacetate, mp 129-130° [lit. mp 134° (Weast, 1968)].

Preparation of 4-hydroxycarbaryl glucoside (III) and 5-hydroxycarbaryl glucoside (IV) - (1). 4-(N-Methylcarbamoyloxy)-1-naphthyl-beta-D-glucopyranoside (III). A mixture of 2.62 g (0.0048 mol) of 4-hydroxycarbaryl glucoside tetraacetate (I) and 300 ml of anhydrous methanol was cooled to 4°, and 1.04 ml (0.00048 mol) of a 0.463 N barium methylate solution (Mitchell, 1941) was added. The reaction temperature was maintained at 4°, and after 7 hr of intermittent shaking a yellow solution was obtained. The solution was neutralized by the addition of an exact equivalent of standard 1N sulfuric acid (0.48 ml). Removal of the almost colloidal barium sulfate which formed was facilitated by the addition of charcoal followed by filtration through Celite.

Evaporation of the filtrate left a syrupy residue which solidified upon being stirred with diethyl ether (350 ml). Filtration gave 1.45 g (80%) of (III), mp 204-208°. Two recrystallizations from methanol/diethyl ether (1:2) afforded 0.63 g (35%) of (III) as off-white crystals, mp 216-218°. (2). 5-(N-Methylcarbamoyloxy)-1-naphthyl-beta-D-glucopyranoside (IV). A mixture of 1.12 g (0.00205 mol) of 5-hydroxycarbaryl glucoside tetraacetate (II) and 70 ml of anhydrous methanol was cooled to 4°, and 0.44 ml (0.000205 mol) of a 0.463 N barium methylate solution was added. The reaction temperature was maintained at 4°, and after 3 hr of intermittent shaking a light pink solution was obtained.

The reaction mixture was processed in a manner similar to that described above for 4-hydroxycarbaryl glucoside (III); 0.57 g &73%) of (IV) as a colorless solid, mp 178-182°, was obtained. A single recrystallization from methanol/diethyl ether (1:3) gave 0.31 g (40%) of (IV) as off-white crystals, mp 197-200°.

Preparation of 4-hydroxynaphthyl glucoside (V) and 5-hydroxynaphthyl glucoside (VI) - (1) 4-Hydroxy-1-naphthyl-beta-D-glucopyranoside (V). A solution of 0.50 g (0.0013 mol) of 4-hydroxycarbaryl glucoside (III), 20 ml of methanol, and 4.8 ml (0.0013 mol) of a 0.277 N barium hydroxide solution was kept at room temperature for 1/2 hr. The solution was neutralized with 0.71 ml (0.0014 mol) of a 2.02 N oxalic acid solution. The mixture was cooled to 4° to allow for complete precipitation of the barium oxalate. Charcoal was added and the cooled mixture was filtered through Celite. The filtrate was evaporated to give 0.42 g of a brown solid, mp 235-240°. A single recrystallization from methanol gave 0.13 g. (2). 5-Hydroxy-1-naphthyl-beta-D-glucopyranoside (VI). A solution of 0.50 g (0.0013 mol) of 5-hydroxycarbaryl glucoside (IV), 20 ml of methanol, and 4.8 ml (0.0013 mol) of a 0.277 N barium hydroxide solution was kept at room temperature for 1/2 hr. The reaction mixture was processed in the same manner as described above for 4-hydroxynaphthyl glucoside (V); 0.45 g of a tan solid, mp 224-228°, was obtained. Recrystallization from methanol gave 0.23 g (53%) of (VI) as tan crystals, mp 232-234°. A second recrystallization from isopropanol gave the analytical sample, mp 236-239°.

Preparation of methyl [5-(N-methylcarbamoyloxy)-1-naphthyl-tri-O-acetyl-beta-D-glucopyranosid] uronate (VII)- A mixture of 5.20 g (0.024 mol) of 5-hydroxycarbaryl, 7.52 g (0.020 mol) of methyl tetra-O-acetyl-beta-D-glucopyranuronate, 0.28 ml (0.0022 mol) of boron trifluoride ether complex (98%), and 250 ml of anhydrous benzene was refluxed with stirring for 16 hr. The dark red mixture was cooled to 4° and the unreacted 5-hydroxycarbaryl was filtered.

The filtrate was washed successively with a cold solution of 0.5 N sodium hydroxide (2 x 60 ml) and ice water (4 x 100 ml), then dired over magnesium sulfate, and concentrated. The residue crystallized upon addition of 50 ml of diethyl ether and was filtered to give 47. g of an off-white solid. Tlc (silica gel; 7:3 benzene-ether) showed this solid to a mixture of methyl tetra-O-acetyl-beta-D-glucopyranuronate and (VII). The solid was dissolved in 50 ml of acetone, and 6 g of Florisil (60/100 mesh) was added. The mixture was concentrated to dryness by rotary evaporation and placed in a glass column containing 155 g of Florisil. The column was eluted with 2 l. of diethyl ether. The ether eluate was evaporated to give 2.5 g (33%) of unreacted methyl tetra-O-acetyl-beta-D-glucopyranuronate, mp 171-175° [lit. mp 176.5-178° (Bollenback et al., 1955)]. Further elution of the column with 1.2 l. of a solution of 10% acetone in benzene, followed by evaporation of the eluate gave 1.77g (16%) of VII, mp 167-172°. Recrystallization of a small amount of this solid from isopropanol gave the analytical sample, as off-white crystals, mp 174-177°.

Toxicity studies - The acute toxicity of 4- and 5-hydroxycarbaryl and their beta-D-O-glucosides to male white mice (Swiss-Webster, 20 g) was evaluated by intraperitoneal injections in 0.1 ml dimethylsulfoxide. The animals were observed for 3 weeks after treatment.

Results and Discussion

To synthesize beta-D-O-glucosides of 4- and 5-hydroxycarbaryl, the N-methylcarbamoyl group in 4- and 5-hydroxycarbaryl was conveniently used as a protecting group. This was necessary since the condensation of 1,5-naphthalenediol with beta-D (+) glucose pentaacetate and catalytic amounts of boron trifluoride ether complex in anhydrous benzene at room temperature for 4 days gave the diglucoside of 1,5-naphthalenediol in low yield. The diglucoside was identified by its ir and nmr spectra.

The boron trifluoride method was evaluated since it had been used in trace amounts as a catalyst for the condensation of beta-D (+) glucose pentaacetate with various phenols (Bretschneider and Beran, 1949). For example, 1-naphthyl-beta-D-glucoside tetraacetate was obtained in 58% yield when the reaction was carried out in benzene at room temperature for two days. In the current study the reaction of 5-hydroxycarbaryl with beta-D (+) glucose pentaacetate and catalytic amounts of boron trifluoride ether complex in anhydrous benzene at room temperature for 5 days gave an 8% yield of 5-hydroxycarbaryl glucoside tetraacetate (II). Since the yield was low, the separation of (II) from the starting material, glucose pentaacetate, was difficult. It was subsequently found that by refluxing the reaction mixture for 1 hr a 23% yield of (II) could be obtained; refluxing for longer periods of time, up to 24 hr, did not improve the yield. This modification, besides almost tripling the yield, significantly reduced the reaction time, and also permitted the facile removal of glucose pentaacetate from (II) by its extraction with diethyl ether. 4-Hydroxycarbaryl glucoside tetraacetate (I) was obtained in a similar manner from 4-hydroxycarbaryl in 19% yield. (I) and (II) were identified by their elemental analyses, and by their ir, nmr, and mass spectra.

The ir spectra of (I) and (II) were consistent with the proposed structures. Although these spectra were similar, they were distinguished by the out-of-plane CH bending vibrations of the naphthyl ring. The frequency of the CH out-of-plane vibration is determined by the number of adjacent hydrogens on the ring (Williams and Fleming, 1966). Therefore, the ir spectra of (II) contained a single strong absorption at 791 cm^{-1}, while the ir spectra of (I) gave several absorptions at 839, 778 and 766 cm^{-1} due to this vibration. The nmr spectra of (I) and (II) were also consistent with the proposed structures. These spectra were taken in acetone-d_6, and upon shaking with deuterium oxide the NH resonance dissappeared and the N-methyl group resonated as a singlet.

The mass spectra of (I) and (II) both contained a weak molecular ion at m/e 547, and a low intensity ion at m/e 490 due to the "parent" ion minus CH_3NCO. An intense peak corresponding to the CH_3NCO fragment at m/e 57 was also present in both spectra. The base peak in each spectra, which occurred at m/e 43, was due to the acylium ion ($CH_3CO^{\oplus}$). The peaks in the mass spectrum of (II) that arose from the aglycone moiety were similar to those obtained in the mass spectrum of (I).

The acetylated glucosides (I) and (II) were deacetylated with catalytic amounts of barium methoxide in anhydrous methanol at 4°. These reaction conditions permitted the removal of the acetate groups without causing significant hydrolysis of the base labile carbamate group. Therefore, 4-hydroxycarbaryl glucoside (III) was obtained in 80% yield by the reaction of (I) with barium methoxide in anhydrous methanol for 7 hr at 4°. 5-Hydroxycarbaryl glucoside (IV) was obtained in 73% yield by the similar hydrolysis of (II). (III) and (IV) were identified by their elemental analyses, and by their ir, nmr, and mass spectra.

The ir spectra of (III) and (IV) were similar, but as with the acetylated glucosides (I) and (II), they could be distinguished by their respective aromatic protons out-of-plane deformation. The nmr spectra of (III) and (IV) were also similar, but again they were differentiated by the 2 proton singlet in the aromatic region of the nmr spectrum of (III).

The mass spectra of (III) and (IV) proved to be very interesting. Even though glucosides are very polar organic compounds, a weak molecular ion was obtained for (III) and (IV) at m/e 379. A weak ion at m/e 322, due to the "parent" ion minus CH_3NCO, was also present in (III) and (IV). The CH_3NCO fragment was detected as a relatively intense fragment at m/e 57 in both spectra. The base peak in the mass spectra of (III) and (IV) occurred at m/e 160 and was due to 1,4-naphthalenediol and 1,5-naphthalenediol respectively. The corresponding sugar moiety was obtained at m/e 163 ($C_6H_{11}O_5^{\oplus}$). The latter peak was of low intensity, but evidence was obtained for an intermolecular rearrangement.

It was shown that transcarbamylation had occurred between the phenolic oxygen of the naphthalene ring and one of the hydroxyl oxygens on the sugar moiety. A peak, which was approximately 5 times as intense as the 163 peak, was obtained at m/e 220 in the spectra of (III) and (IV). High resolution mass spectral analysis determined the elemental composition of this peak to be either $C_8H_{14}O_6N_1$ or $C_{16}H_{12}O_1$. The latter compoistion was discarded on the basis of the chemical strucutre of the glucosides, and therefore the peak at 220 was of elemental composition $C_8H_{14}O_6N_1$ (theoretical mass 220.0821; actual mass, (III) 220.089 ± 0.010, (IV) 220.080 ± 0.010). This elemental composition was consistent with carbamylated glucose. It should be noted that the N-methylcarbamoyl group could be on any one of the four hydroxyl oxygens of the sugar. Further evidence for this structure was obtained when the mass spectrum of a mixture of 1-naphthyl-beta-D-glucoside and 5-hydroxycarbaryl were taken separately, neither spectrum contained an ion at this mass.

Samples of (III), mp 204-208°, and (IV), mp 178-182°, prepared by the barium methoxide procedure, were shown by aluminum oxide tlc to contain an impurity which traveled just below (III) and (IV). These impurities were identified as their respective decarbamylated products 4-hydroxy-1-naphthyl-beta-D-glucopyranoside (V) and 5-hydroxy-1-naphthyl-beta-D-glucopyranoside (VI). The latter compounds were independently synthesized by the hydrolysis of (III) and (IV) with a barium hydroxide solution in methanol at room temperature. (V) and (VI) were identified by their elemental analyses, tlc, and their ir spectra, which was similar to the ir spectra of (III) and (IV), but did not contain a carbonyl streching frequency at approximately 1720 cm^{-1}. The instability of the carbamate ester in basic solutions was demonstrated when approximately 50% hydrolysis was obtained with catalytic amounts of sodium methoxide in methanol at room temperature.

The reaction of 5-hydroxycarbaryl with methyl tetra-O-acetyl-beta-D-glucopyranouronate and catalytic amounts of boron trifluoride ether complex in refluxing anhydrous benzene for 16 hr gave a 15% yield of methyl [5-(N-methylcarbamoyloxy)-1-naphthyl-tri-O-acetyl-beta-D-glucopyranosid] uronate (VII). This reaction was significant since aryl-beta-D-glucuronides are more commonly prepared by the condensation of methyl (tri-O-acetyl-alpha-D-glucopyranosyl bromide) uronate with a phenol in the presence of an acidic metal catalyst (Bollenback et al., 1955; Coffey, 1967; Conrow and Bernstein, 1971), or by the fusion of the acetylated methyl ester sugar with a phenol in the presence of an acidic catalyst at reduced pressures (Bollenback et al., 1955). The structural assignment for (VII) is supported by its elemental analysis, and by its ir, nmr, and mass spectra.

(VII) was deacetylated with catalytic amounts of barium methoxide in methanol at 4°. However, attempts at hydrolyzing the methyl ester without hydrolyzing the carbamate ester were not successful. The use of barium hydroxide at 4° hydrolyzed the carbamate group along with the methyl ester. The mass spectrum of the methyl ester of the beta-D-glucuronide of 5-hydroxycarbaryl had a weak molecular ion at m/e 407. The base peak was at m/e 160 (1,5-naphthalenediol). The mass spectrum also showed a peak at m/e 248, which corresponded to the thermal rearrangement peak at m/e 220 in the mass spectra of (III) and (IV). Thermal intermolecular rearrangements of this type may prove quite common in the mass spectra of carbamate glucosides, and therefore may be useful in their identification.

The attempted preparation of the beta-D-O-glucoside of N-hydroxymethylcarbaryl by a procedure similar to that used for the preparation of 4- and 5-hydroxycarbaryl glucoside was unsuccessful. Acetylation was not accomplished since N-hydroxymethylcarbaryl was not stable in benzene solutions containing catalytic amounts of boron trifluoride. Silica gel tlc showed that it decomposed almost immediately in the acidic mixture to at least four products.

Preliminary experiments showed that the beta-D-O-glucoside of 3-hydroxycarbofuran (2,3-dihydro-2,2-dimethylbenzofuranyl-7 N-methylcarbamate) could not be synthesized using the procedure described herein.

3-Hydroxycarbofuran, beta-D (+) glucose pentaacetate, and catalytic amounts of boron trifluoride ether complex were refluxed in anhydrous benzene for 16 hr. The reaction mixture was processed in a manner similar to that described for 4- and 5-hydroxycarbaryl glucoside tetraacetates. The major product isoalted, mp 235-240° dec., did not contain the acetylated sugar moiety. Its elemental analysis, and its ir and nmr spectra indicated that it was a self-condensation product of 3-hydroxycarbofuran.

The toxicity of 4- and 5-hydroxycarbaryl and their respective beta-D-O-glucosides (III) and (IV) to mice are reported in Table 70. In all cases mortalities occurred within 2 hr after administration of the test compounds, with no further mortalities occurring during an observation period of 3 weeks. No mortalities occurred in the control mice. As expected, glucoside formation greatly decreased the toxicity of 4- and 5-hydroxycarbaryl. However, even though the aglycones differed only slightly in toxicity, their glucosides showed a relatively greater difference. This could possibly indicate that some of the toxicity of (IV) was due to the intact glucoside, and not entirely the result of cleavage of the conjugated form to yield the toxic aglycone. However, it is possible that 5-hydroxycarbaryl glucoside is cleaved faster than its 4-hydroxy analog in mice, resulting in an effectively higher concentration of 5-hydroxycarbaryl. Further studies using the synthetic conjugates will enhance the elucidation of this point and allow the general toxicological nature of the carbaryl glucosides to be more completely defined.

TABLE 70. THE TOXICITY OF 4- AND 5-HYDROXYCARBARYL AND THEIR RESPECTIVE BETA-D-O-GLUCOSIDES (III) AND (IV) TO MICE[a]

Compound	LD_{50},mg/kg[b]
4-hydroxycarbaryl	50
4-hydroxycarbaryl glucoside (III)	1550
5-hydroxycarbaryl	55
5-hydroxycarbaryl glucoside (IV)	950

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[a] Compounds were administered by intraperitoneal injection using dimethylsulfoxide as carrier.

[b] Mortality was recorded after 24 hours.

IV REFERENCES

1. Andrawes, N. R., H. W. Dorough, and D. A. Lindquist. Degradation and elimination of Temik in rats. J. Econ. Entomol. 60: 879-87 (1967).

2. Bartley, W. J., N. R. Andrawes, E. L. Chancey, W. P. Bagley, and H. W. Spur. The metabolism of aldicarb pesticide in the cotton plant. J. Agr. Food Chem. 18: 446 (1970).

3. Baron, R. L. and J. D. Doherty. Metabolism and excretion of insecticide (6-chloro-3,4-dimethyl N-methylcarbamate) in the rat. J. Agr. Food Chem. 15: 830 (1967).

4. Baron, R. L., N. J. Palmer, R. Ross, J. Doherty, and W. C. Jacobson. Distribution of radioactivity in milk resulting from oral administration of C-labeled carbaryl. J. Ass. Offic. Anal. Chem. 51: 32 (1968).

5. Baron, R. L., J. A. Sphon, J. T. Chen, E. Lustig, J. D. Doherty, E. A. Hansen, and S. M. Kolbye. Confirmatory insolation and identification of a metabolite of carbaryl in urine and milk. J. Agr. Food Chem. 17: 883 (1969).

6. Bollag, J. M., and S. Y. Liu. Biodegradation of Sevin by soil microbes. Bact. Proc. ASM p. 9 (1970).

7. Bollag, J. M., and S. Y. Lui. Fungal transformation of the insecticide carbaryl. Bact. Proc. ASM p. 16 (1971).

8. Bollen, W. B. Interactions between pesticides and soil microorganisms. Ann. Rev. Microbiol. 15: 69-92 (1961).

9. Bollenback, G. N., J. W. Long, D. G. Benjamin, and J. A. Lindquist. The synthesis of Aryl-D-glucopyranosiduronic acids. J. Amer. Chem. Soc. 77: 3310 (1955).

10. Boose, R. B., and L. C. Terriere. Quantitative aspects of the detoxication of naphthalene by resistant and susceptible houseflies. J. Econ. Entomol. 60: 580 (1967).

11. Bretschneider, H., and K. Beran. Uberfuhuany un-und zweiwertiger phenole in acetylierte beta-D-glucoside mit beta-pentaacetyl-D-glucose und borfluorid BF_3 as catalyst. Monatsh. 80: 262 (1949).

12. Brodie, B. B., J. R. Gillette, and B. N. LaDu. Enzymatic metabolism of drugs and other foreign compounds. Annu. Rev. Biochem. 27: 427-54 (1958).

13. Casida, J. E. Mixed-function oxidase involvement in the biochemistry of insecticide synergists. J. Agr. Food Chem. 18: 753-72 (1970).

14. Chung, R. A., I. L. Huang, and R. W. Brown. Studies of DNA, RNA and protein synthesis in He La S cells exposed to DDT and dieldrin. J. Agr. Food Chem. 15: 497 (1967).

15. Claborn, H. V., R. H. Roberts, H. D. Mann, M. C. Bowman, M. C. Ivey, C. P. Weidenbach, and R. D. Radleff. Residues in body tissues of livestock sprayed with Sevin or given Sevin in the diet. J. Agr. Food Chem. 11: 74 (1963).

16. Coffey, S. "Rodd's Chemistry of Carbon Compounds". 2nd ed., Vol. 1, Elsevier Publishing Co., Amsterdam, 1967 p. 335.

17. Conrow, R. B., and S. Bernstein. Steroid conjugates. VI. An Improved koenigs-knorr synthesis of aryl glucuronides using cadmium

carbonate, a new and effective catalyst. J. Agr. Food Chem. 36: 863 (1971).

18. Coppedge, J. R., D. A. Lindquist, D. L. Bull, and H. W. Dorough. Fate of Temik. J. Agr. Food Chem. 15: 902 (1967).

19. Culver, D. J., T. Lin, and H. W. Dorough. Effect of monoamine oxidase inhibitors on carbaryl metabolism. J. Econ. Entomol. 63: 1369-70 (1970).

20. Dorough, H. W. Carbaryl-C^{14} metabolism in a lactating cow. J. Agr. Food Chem. 15: 261 (1967).

21. Dorough, H. W. Metabolism of Furadan in rats and houseflies. J. Agr. Food Chem. 16: 319-25 (1968).

22. Dorough, H. W. Metabolism of insecticidal methylcarbamates in animals. J. Agr. Food Chem. 18: 1015-22 (1970).

23. Dorough, H. W. Carbaryl residues in milk and meat of dairy animals. Pesticide Terminal Residues, Supplement to Pure and Applied Chem. p. 173 (1971).

24. Dorough, H. W., and J. E. Casida. Nature of certain carbamate metabolites of the insecticide Sevin. J. Agr. Food Chem. 12: 294 (1964).

25. Dorough, H. W., and G. W. Ivie. Temik-S^{35} metabolism in a lactating cow. J. Agr. Food Chem. 16: 460 (1968).

26. Dorough, H. W., H. M. Mehendale and T. Lin. Modification of carbaryl metabolites in rats with monoamine oxidase inhibitors. J. Econ. Entomol. 65: 958 (1972).

27. Dorough, H. W. and O. G. Wiggins. Nature of the water-soluble metabolites of carbaryl in bean plants and their fate in rats. J. Econ. Entomol. 62: 49-53 (1969).

28. Durden, J. A., W. J. Bartley, and J. F. Stephen. The preparation of standards for aldicarb (Temik) metabolism study. J. Agr. Food Chem., 18: 454 (1970).

29. Ellman, G. L., H. D. Courtney, V. Anders, and R. M. Featherstone. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7: 88-95 (1961).

30. Fieser, L. F. "Experiments in Organic Chemistry", 3rd ed., D. C. Heath and Company, Boston, 1957. p 289.

31. Fouts, J. R. The stimulation and inhibition of hepatic microsomal drug-metabolizing enzymes with special reference to effects of environmental contaminants. Tox. Appl. Pharmacol. 17: 804 (1970).

32. Gillett, J. W. and T. M. Chan. Cyclodiene insectiicdes as inducer substances and inhibitors of microsomal epoxidation. J. Agr. Food Chem. 16: 590 (1968).

33. Gyrisco, F. G., D. J. Lisk, S. N. Fertig, E. W. Huddleston, F. N. Fox, R. F. Holland, and G. W. Trinberger. The effects of feeding high levels of Sevin on residue, flavor, and odor of the milk of dairy cattle. J. Agr. Food Chem. 8: 409 (1960).

34. Hargreaves, T. The effect of monoamine oxidase inhibitors on conjugation. Experientia (Basel) 24: 157-8 (1968).

35. Hart, L. G., and J. R. Fouts. Studies of the possible mechanisms by which chlordane stimulates hepatic microsomal drug metabolism in the rat. Arch. Expt. Pathol. Pharmacol. 249: 486 (1965).

36. Hayes, W. J., Jr. "Clinical Handbook on Economic Poisons." U. S. Public Health Service Publication No. 476. 147 pages (1963).

37. Hicks, B. W., H. W. Dorough, and R. B. Davis. Fate of carbofuran in laying hens. J. Econ. Entomol. 63: 1108 (1970).

38. Jeffay, H., and J. Alvarez. Liquid scintillation counting of carbon-14 use of ethanolamine-ethyleneglycol monomethylether-toluene. Anal. Chem. 33: 612 (1961).

39. Kelly, R. G., E. A. Peets, S. Gordon, and D. A. Buyske. Determination of C-14 and H^3 in biological samples by Schoniger combustion and liquid scintillation techniques. Anal. Biochem. 2: 267 (1961).

40. Knaak, J. B., M. J. Tallant, W. J. Bartley, and L. J. Sullivan. The metabolism of carbaryl in the rat, guinea pig, and man. J. Agr. Food Chem. 13: 537 (1965).

41. Kuhr, R. J. Metabolism of carbamate insecticide chemicals in plants and insects. J. Agr. Food Chem. 18: 1023-30 (1970).

42. Kuhr, R. J., and J. E. Casida. Persistant glucosides of metabolites of methyl-carbamate insecticide chemicals formed by hydroxylation in bean plants. J. Agr. Food Chem. 15: 815 (1967).

43. Liu, S. Y., and J. M. Bollag. Carbaryl decomposition to 1-naphthyl carbamate by Aspergillus terreus. Pest. Biochem. Phys. 1: 366 (1971a).

44. Liu, S. Y., and J. M. Bollag. Metabolism of carbaryl by a soil fungus. J. Agr. Food Chem. 19: 487 (1971b).

45. Lowry, O. H., N. J. Rosenbrough, A. L. Furr, and R. J. Randall. Protein measurement with Folin phenol reagent. J. Biol. Chem. 193: 265 (1951).

46. Mehendale, H. M., and H. W. Dorough. Conjugative metabolism and action of carbamate insecticides. Proc. 2nd Internal. Cong. Pest. Chem. Tel-Aviv, Israel, Feb. 21-27 (1971).

47. Mehendale, H. M., and H. W. Dorough. Glucuronidation mechanisms in the rat and their significance in the metabolism of insecticides. Pestic. Biochem. Physiol. 1: 307 (1972).

48. Metcalf, R. L., T. R. Fukuto, C. Collins, K. Borck, J. Burke, H. T. Reynolds, and M. F. Osman. Metabolism of 2-methyl-2-(methylthio)-propionaldehyde O-(methylcarbamoyl)-oxime (Temik) in plants and insects. J. Agr. Food Chem. 14: 579 (1966).

49. Miles, J. R. W., C. M. Tu, and C. R. Harris. Metabolism of heptachlor and its degradation products by soil microorganism. J. Econ. Entomol. 62: 1334 (1969).

50. Mumma, R. O., S. Khalifa, and R. H. Hamilton. Spectroscopic identification of metabolites of carbaryl in plants. J. Agr. Food Chem. 19: 445 (1971).

51. Oonnithan, E. S. and J. E. Casida. Oxidation of methyl- and dimethylcarbamate insecticide by microsomal enzymes and anti-cholinesterase activity of the metabolites. J. Agr. Food Chem. 16: 28 (1968).

52. Parke, D. V. "The Biochemistry of Foreign Compounds." pp. 269 Pergamon, London, 1968.

53. Reed, D. K., K. Goto, and C. H. Wang. A direct radioisotopic assay for acetylcholinesterase. Annal. Biochem. 16: 59 (1966).

54. Simpson, D. R., D. L. Bull, and D. A. Lindquist. A semimicro-technique for the estimation of cholinesterase activity in boll weevils. Annals. Entomol. Soc. Am. 57: 367-71 (1964).

55. Stanier, R. Y. Simultaneous adaptation. A new technique for the study of metabolic pathways. J. Bacteriol. 54: 339-48 (1947).

56. Stockley, I. H. Interaction of monoamine oxidase inhibitors with food and drugs. Part I. Pharm. J. 203: 147-73 (1969a).

57. Sullivan, L. J., J. M. Eldridge, J. B. Knaak, and M. J. Tallant. 5,6-Dihydro-5,6-dihydroxycarbaryl glucuronide as a significant metabolite of carbaryl in the rat. J. Agr. Food Chem. 20: 980 (1972).

58. Tewfik, M. S., and Y. A. Hamdi. Decomposition of Sevin by a soil bacterium. Acta. Microbiol. Polonica Ser B. 19: 133-5 (1970).

59. Timmerman, J. A., H. W. Dorough, J. R. Buttram, and B. W. Arthur. In vitro stability and recovery of insecticides from milk. J. Econ. Entomol. 54: 441 (1961).

60. Wasserman, M. Effects of organochlorine insecticides on body defense systems. Ann. Rev. N. Y. Acad. Sci. 160: 393 (1969).

61. Weast, R. C. "Handbook of Chemistry and Physics". 49th ed., The Chemical Rubber Co., Ohio, 1968, p c-336.

62. Wiggins, O. G., and M. H. J. Weiden. Union Carbide Corp. South Charleston, W. Va. Private communication (1969).

63. Williams, R. T. Metabolic fate of foreign compounds. Arch. Environ. Health. 7: 612-20 (1963).

64. Williams, S., and P. A. Mills. Residues in milk of cows fed rations containing low concentrations of five chlorinated hydrocarbon pesticides. J. Assoc. Offic. Agr. Chem. 47: 1124-28 (1964).

65. Whitehurst, W. E., E. T. Bishop, F. E. Critchfield, F. G. Gyrisco, E. W. Huddleston, H. Arnold, and D. J. Lisk. The metabolism of Sevin in dairy cows. J. Agr. Food Chem. 11: 167 (1963).

V PUBLICATIONS

1. Dorough, H. W., R. B. Davis and G. W. Ivie, 1970. Fate of Temik-C^{14} in lactating cows during a 14-day feeding period. J. Agr. and Food Chem. 18 (1): 135-42.
2. Hicks, B. W., H. W. Dorough, and R. B. Davis. 1970. Fate of carbofuran in laying hens. J. Econ. Entomol. 63 (4): 1108-11.
3. Culver, D. J., T. Lin and H. W. Dorough. 1970. The effect of monoamine oxidase inhibitors on carbaryl metabolism. J. Econ. Entomol. 63 (4): 1369-70.
4. Dorough, H. W. 1970. Metabolism of insecticidal methylcarbamates in animals. J. Agr. Food Chem. 18 (6): 1015-22.
5. Dorough, H. W. 1971. Carbaryl residues in milk and meat of dairy animals. Proceedings of the "International Symposium on Pesticide Terminal Residues". Tel-Aviv, Israel, Feb. 17-19, 1971.
6. Mehendale, H. M. and H. W. Dorough. 1971. Conjugative metabolism and action of carbamate insecticides. Proceedings of the "Second International Congress of Pesticide Chemistry" Tel-Aviv, Israel, Feb. 21-27, 1971.
7. Mehendale, H. M. and H. W. Dorough. 1972. In vitro glucosylation of 1-naphthol by insects. J. Insect Physiol. 18: 981-90.
8. Mehendale, H. M. and H. W. Dorough. 1972. Glucuronidation mechanisms in the rat and their significance in metabolism of insecticides. Pest. Biochem. and Physiol. 1: 307-18.
9. Hicks, B. W., H. W. Dorough and H. M. Mehendale. 1972. Metabolism of aldicarb pesticide in laying hens. J. Agr. Food Chem. 20 (1): 151-56.
10. Mehendale, H. M., R. F. Skrentny and H. W. Dorough. 1972. Oxidative metabolism of aldrin by subcellular root fractions of several plant species. J. Agr. Food Chem. 20: 398-402.
11. Dorough, H. W., H. M. Mehendale and T. Lin. 1972. Modification of carbaryl metabolism in rats with monoamine oxidase inhibitors. J. Econ. Entomol. 65 (4): 958-62.

12. Cardona, R. A. and H. W. Dorough. 1973. Syntheses of the β-D-O-glucosides of 4- and 5-hydroxy-1-naphthyl N-methylcarbamate. J. Agr. Food Chem. (In Press).

13. Lin, T. H. and H. W. Dorough. Influence of selected insecticides on carbaryl metabolism in rats (In Preparation).

14. Lin, T. H., El-Shourbagy, N. A., and H. W. Dorough. Fate of 3-hydroxycarbofuran and its glucoside and glucuronide in rats. (In Preparation).

15. El-Shourbagy, N. A. and H. W. Dorough. Factors influencing glycoside conjugation in insects and mammals. (In Preparation).

16. Dorough, H. W., J. P. McManus and S. S. Kumar. Chemical and metabolic characteristics of 1-naphthyl glucoside. (In Preparation).

THESIS

1. Hicks, B. W. "Fate of Furadan and Temik in Laying Hens". Ph.D. 1970. Texas A&M University, College Station, Texas.

2. Lin, T. H. "Influence of Selected Biologically Active Chemicals on the Mammalian Metabolism of Carbamate Insecticides." Ph.D. 1972. University of Kentucky, Lexington, Kentucky.

3. El-Shourbagy, N. A. "Glycoside Conjugation in Insects and Mammals." M.S. 1972. University of Kentucky, Lexington, Kentucky.

4. Rodriguez, L. D. "Studies in Degradation of Carbaryl by Soil Microorganisms." Ph.D. 1973. University of Kentucky, Lexington, Kentucky.

VI SUMMARY

The current project "Metabolism of Carbamate Insecticides" was initiated in 1965 and was originally funded by the National Institute of Health. Detailed progress reports and requests for renewal were submitted in April 1967 and in December 1969. The present report covers research conducted under EPA Grant Number R-802005 during the period of September 1, 1970 through August 31, 1973.

METABOLISM

Aldicarb

Increasing the number of days, up to 14, that cows were administered aldicarb [2-methyl-2(methylthio-C^{14}) propionaldehyde O-(methylcarbamoyl) oxime] at levels of 0.12, 0.6, and 1.2 ppm in the diet did not alter the magnitude and nature of residues eliminated daily in the milk, urine, and feces. Parts per million total aldicarb equivalents in the milk were approximately 1/100 that level of insecticide in the feed. About 15% of the radioactive residues in the milk was aldicarb sulfone and about 4% was aldicarb sulfoxide. The remaining was hydrolytic products and compounds of unknown identity. Percentages of the doses eliminated in the milk, urine, and feces were 1, 92, and 3 respectively. Total aldicarb equivalents in the liver were 29, 123, and 164 ppb for the three treatment rates, respectively, when the animals were slaughtered 18 hours after the last treatment. Twenty-six other tissue samples contained either much lower quantities of residues or none at all.

Aldicarb metabolism in laying hens was invetigated and the nature and levels of residues in the eggs and tissues were determined. Single oral doses of aldicarb and/or aldicarb sulfone at 0.7 mg/kg were excreted rapidly, with 75% of the doses in the feces by 24 hr. A large portion of the feces metabolites (50-60%) was as water-soluble materials, 10% as unextractables, and the remainder primarily as known hydrolytic products of aldicarb. Only minute quantities were as toxic carbamate compounds. Aldicarb equivalents in eggs reached a maximum of 0.18 ppm on the day after treatment but had declined to 0.01 ppm by 10 days. In muscle tissues, residues of 0.2 to 0.3 ppm 6 hr after treatment declined to 0.01 ppm or less by 10 days. Residue levels in the liver and kidney were about twice those in the muscle tissue. The nature of the aldicarb metabolites in the eggs and tissues was similar to that in the feces. Aldicarb in the diet of hens for 21 days did not appear to alter the fate of the carbamate in the birds when compared to that when single oral doses were administered.

Forty and 80% of a topically applied dose of aldicarb was absorbed by boll weevils and houseflies during the first hour after treatment. Complete disappearance from the surface of flies and weevils occurred 6 and 24 hours post-treatment, respectively. The major metabolic pathway in these species appeared to be one of oxidation with both the sulfoxide and sulfone derivatives of aldicarb detected in the insects. Aldicarb sulfoxide is a potent cholinesterase inhibitor and, as such, probably contributes significantly to the insecticidal activity of the parent carbamate compound.

Carbaryl

Cows fed on a diet containing 10, 30 or 100 ppm carbaryl-naphthyl-^{14}C for 14 days eliminated daily about 0.2 percent of the consumed doses in the milk, 5-10 percent in the feces and 70-85 percent in the urine. Average daily levels of carbaryl-^{14}C equivalents in the milk for the three feeding levels were 0.02, 0.07 and 0.28 ppm respectively. Residue levels in the tissues after 14 days of feeding the three levels of carbaryl were as follows: kidney, 0.10, 0.53, 1.00; liver, 0.03, 0.10, 0.41; lung, 0.02, 0.06, 0.21; muscle, 0.01, 0.03, 0.10; heart, 0.01, 0.04, 0.10; blood 0.01, 0.04, 0.14; fat, 0.0, 0.02, 0.02. The chemical nature of the residues in the milk were determined. Extraction with acetonitrile-water-chloroform was the desired solvents for extracting the tissues. Homogenation in water or phosphate buffer and centrifugal fractionation showed that 15 to 20% of the radiocarbon in the liver and kidney remained in the 15,000g pellet. Centrifugation of the 15,000g supernatant at 105,000g resulted in only trace amounts of radiocarbon in the precipitant fraction. After extraction of the precipitants with acetonitrile and water, the solids from the liver contained from 7 to 10% of the total ^{14}C in the whole tissue; the solids from the kidney contained about 5%. Treatment of the precipitants with acid (3N HCl and reflux for 3 hrs) prior to extraction did not significantly improve the extractability of the ^{14}C-residues. The supernatant fractions formed a precipitant fraction when acetonitrile-chloroform was added. Distribution, % of ^{14}C in tissue, in the solids, water phase, and organic solvent phase of the supernatant extracts was as follows: Liver - 40, 20, and 21; Kidney - 13, 35, and 31. Comparable values when the supernatant were treated with acid were: Liver - 36, 23, and 26; Kidney - 6, 39 and 37.

The degradation of carbaryl was studied with three samples of Maury soil: Untreated soil with no recorded pesticide treatment; Carbaryl-treated, which had received 4 lbs/A carbaryl granules six months prior to sampling; and Mixed Pesticide-treated, which had been subjected to various pesticides for 15 years. These soils, non-sterile and autoclaved, were incubated under controlled conditions for periods up to 120 days with added carbaryl-1-naphthyl-1-^{14}C, 1-naphthyl-1-^{14}C and polar water-soluble metabolites of carbaryl produced in bean plants. Total radioactivity in soil preparations to which carbaryl or polar water-soluble metabolites had been added disappeared much faster from non-sterile than from autoclaved soil, indicating biological attack. Of the non-sterile soils, dissipation was fastest in Carbaryl-treated, where 44 percent of the radiocarbon was lost after 48 hours incubation at 27°C, 85 percent relative humidity. Organosoluble radioactivity in soils incubated with carbaryl was composed entirely of the parent compound. Water-soluble metabolites were present in small amounts.

The loss in total radioactivity suggested the evolution of volatile metabolites, of which only carbon dioxide could be demonstrated. After a loss of as much as 50 percent during the first 14 days of incubation, radioactivity added as polar water-soluble metabolites persisted in the non-sterile soils thereafter. Tlc analysis indicated that organosoluble fractions of the soil extracts contained aglycones different from those originally present in enzymatic hydrolysates of the polar water-soluble metabolites. Fifty-eight bacterial and fungal isolations were obtained from Mixed Pesticide-treated soil on silica gel with carbaryl as the sole carbon source and 30 such isolations from Carbaryl-treated soil on carbaryl agar. Those capable of attacking carbaryl in culture medium were identified.

Carbofuran

Carbamate-resistant houseflies absorbed carbofuran slower and excreted it faster than a susceptible strain of the insects. This appears to be an important factor in the added tolerance of the resistant flies to carbofuran since both strains metabolized the compounds in an almost identical manner. Susceptible and carbamate-resistant housefly cholinesterase recovered from inhibition with carbaryl and Baygon at the same rate. The degree of inhibition also was the same in both strains, and no difference could be detected in the sensitivity of the enzyme to the carbamate insecticide. Resistant flies, however, did appear to have greater cholinesterase activity than the susceptible insects.

FATE OF CARBAMATE METABOLITES

1-Naphthyl Glucoside

When 1-naphthyl glucoside was administered orally to rats, 67% of the dose was eliminated in the 0-24 hr urine. About 10% of the dose was as 1-naphthol, 24% as 1-naphthyl glucuronide and 10% as 1-naphthyl sulfate. Urine (0-24 hr) of rats treated with 1-naphthol contained 90% of the dose. Only 1% of the dose was as free 1-naphthol, 73% as the glucuronide and 15% as the sulfate. These studies demonstrated tnat glucoside conjugates are not metabolized in the same manner as the aglycones.

3-Hydroxy Carbofuran and its Glycosides

Approximately 60% of a single oral dose of 3-hydroxy carbofuran to rats was eliminated in the urine within 32 hrs. When 3-hydroxy carbofuran glucuronide was administered, 82% of the dose was in the 0-32 hr urine; 91% of a dose of 3-hydroxy carbofuran glucoside was eliminated in the urine during the same time. The percentage of the radiocarbon in the urine which extracted into chloroform were 39, 18 and 10, respectively. 3-Hydroxy carbofuran was the only component of the chloroform extracts. In bean plants, 3-hydroxy carbofuran was rapidly converted to the glucoside form.

INTERACTIONS

Effect of Aldicarb on Methyl Parathion Toxicity

Aldicarb showed only additive toxic effects to mice when administered along with methyl parathion, an organophosphorus insecticide. The oxime hydrolytic product of aldicarb, which is nontoxic, did not alter the toxicity of methyl parathion in any way, nor was the protective action of the antidotes, atropine and 2-PAM, influenced by the addition of the oxime derivative of aldicarb.

Modification of Carbaryl Metabolism with MAOI's

The rate of carbaryl metabolism by rats was decreased by simultaneously administering drugs commonly referred to as monaomine oxidase inhibitors and by prolonged exposure of the animals to one of the drugs in in the drinking water. Conjugative mechanisms of metabolism were affected most by the drugs although oxidative and hydroxylative metabolism were reduced to some degree. Decreasing the rate of metabolism resulted in a slower rate of excretion of the carbamate from rats treated with the drugs. Tranylcypromine at 50 mg/kg, e.g., reduced excretion of carbaryl-^{14}C equivalents from rats by 50% during the 1st 48 hours after treatment. A rat intestine enzyme system, fortified with UDPGA, conjugated 1-naphthol but did not metabolize carbaryl. This in vitro conjugating system was inhibited by the monoamine oxidase inhibitors. The effect of these drugs on conjugation was not sufficient to suggest that they might enhance the action of pesticides under practical conditions. However, the data do demonstrate that more effective inhibitors of conjugation could be important in the safety and efficacy of chemicals used to control insect pests.

Influence of Insecticides on Carbaryl Metabolism

Effects of simultaneous and/or pre-exposure of carbofuran, Ruelene and coumaphos or DDT on the metabolism of carbaryl in rats and the enzymes involved therein were investigated. In addition, the urea and glucoside content, and pH of the rat urine were considered. Radioactive carbaryl when given orally as a single dose (50 mg/kg) was rapidly eliminated in the urine and feces of rats. The excretion was complete by 72 hours after the treatment with approximately 80% in the urine and 10% in the feces of the rats. Simultaneous doses of carbaryl and carbofuran reduced the excretion of carbaryl equivalents by 12%. Ruelene and coumaphos caused a slightly faster excretion of carbaryl equivalents when administered with the carbamate. None of the compounds changed the nature of carbaryl metabolites or changed the amount of carbaryl residues in the body. Similar results were obtained when these insecticides were administered in the diets of rats. Protein content of the liver, kidney and spleen were increased by DDT and to a lesser degree by carbaryl. DDT also enhanced mixed function oxidase enzyme activities in the liver and the kidney, whereas carbaryl did not. DDT increased the in vivo glucuronide and sulfate conjugation of 1-naphthol in rats but not the in vitro conjugation of 1-naphthol by rat liver microsomes. Carbaryl did not affect the conjugating enzyme activity in the rat liver. Exposure of rats to the carbamate and/or organophosphates resulted in increased urea and glucose in the urine, reduced the rate of body weight gains, but had little effect on the pH of the urine.

CONJUGATION

Mechanisms

Optimum conditions for the in vitro glucuronidation of 1-naphthol were established using rat liver and small intestine as enzyme sources. The liver enzyme system was used to study conjugative metabolism, per se, and in conjunction with oxidative metabolism while the intestine enzyme was used to study conjugative metabolism exclusively. Both carbaryl and Banol required oxidative metabolism before conjugation could take place. However, when conjugative metabolism was reduced by limiting the UDPGA concentration, the overall rate of carbamate metabolism was decreased. There was no accumulation of the nonconjugate metabolites although excess $NADPH_2$ was present in the microsome system. Inhibition of the conjugating enzymes was demonstrated using several insecticide synergists, including sulfoxide, piperonyl butoxide, and MGK-264, which are established mixed-function oxidase inhibitors. A number of insecticides at 10^{-3}M concentration had no effect on the in vitro conjugating enzyme systems. Glucuronides of several hydroxylated carbaryl metabolites were synthesized by the enzymes from the two sources but the 5,6-dihydro-dihydroxy analog of carbaryl could not be conjugated.

Glucosylation in insects was investigated using tobacco hornworms (Manduca sexta) as the primary test insect and 1-naphthol-^{14}C as the substrate. Of 6 common co-factors tested, only UDPG was utilized by the conjugating enzyme system. Neither the hornworm nor housefly enzymes could form the glucuronic acid derivative of 1-naphthol using UDPGA.

Centrifugal fractionation of the hornworm homogenates showed that the glucosyltransferase activity was in the 105,000g soluble fraction. In the housefly, the enzyme activity was associated with the 15,000g pellet and to a lesser extent with the 105,000g pellet. In vitro inhibition of the glucosyltransferase by sulphoxide, piperonyl butoxide, and other insecticide synergists were demonstrated.

Factors Influencing Conjugation

The occurrence and comparative activity of glycoside conjugating enzymes were investigated in insect and vertebrate species. All species readily conjugated the naphthol, although there were quantitative differences. Glucosyltransferase activity was greatest in the fat bodies, malphigian tubules and midgut of the tobacco hornworm larvae. In the rat, the comparable enzyme, glucuronlytransferase was most active in the liver, kidney and lungs. Glycoside conjugating enzymes maintained almost 100% of their activity when stored in the intact tissue or in various subcellular fractions at -20° and 0°C for 90 days. TPNH oxidase enzymes lost most of their activity after a few hours of storage. In vitro glucosylation of 1-naphthol by houseflies was not greatly influenced by developmental stage, age of the adult, sex or by acquired resistance to insecticides. The enzymes were not affected by the addition of various insecticides to the reaction mixtures. Glycoside conjugation, like oxidation and hydrolytic metabolism, plays a major role in the detoxication of insecticides and other foreign compounds. The enzymes responsible for glycosylation, however, are unlike these other enzyme systems in that they are virtually unaffected by factors such as age, sex, resistance and other toxicants.

Synthesis of Glycosides

4- and 5-(N-Methylcarbamoyloxy)-1-naphthyl-tetra-O-acetyl-beta-D-glucopyranoside were synthesized by the condensation of the appropriate hydroxy-1-naphthyl-N-methylcarbamate with beta-D (+) glucose pentaacetate in the presence of catalytic amounts of boron trifluoride ether complex. Reaction of the acetylated beta-D-glucosides with barium methoxide gave the corresponding beta-D-glucosides. The ir, nmr, and mass spectra of the beta-D-glucosides and their acetylated analogs are reported and compared. When administered ip to mice 4-hydroxy-1-naphthyl-N-methylcarbamate was 31 times more toxic than its beta-D-glucoside, 5-hydroxy-1-naphthyl-N-methylcarbamate was 17 times more toxic than its beta-D-glucoside. Methyl [5-(N-methylcarbamoyloxy)-1-naphthyl-tri-O-acetyl-beta-D-glucopyranosid] uronate was prepared in a manner similar to the acetylated beta-D-glucosides. The deacetylated methyl ester was prepared by hydrolysis with barium methoxide, however, attempts to demethylate the product while leaving the carbamate ester intact were unsuccessful.

The work conducted on this project has resulted in 11 publications in print, 1 in press and 4 in preparation.